A polymorphism in the promoter is associated with EZH2 expression but not with outcome in advanced pancreatic cancer patients

Aim: EZH2 expression is a prognostic marker in radically resected pancreatic ductal adenocarcinoma (PDAC) patients. Here we investigated its role in locally advanced/metastatic patients, as well as candidate polymorphisms. **Materials & methods:** EZH2 expression and polymorphisms were evaluated by quantitative reverse transcription PCR in 32 laser microdissected tumors, while polymorphisms were also studied in blood samples from two additional cohorts treated with gemcitabine monotherapy (n = 93) or polychemotherapeutic regimens (n = 247). **Results:** EZH2 expression correlated with survival and with the rs6958683 polymorphism in the first cohort of patients, but this polymorphism was not associated with survival in our larger cohorts. **Conclusion:** EZH2 is a prognostic factor for locally advanced/metastatic PDACs, while candidate polymorphisms cannot predict clinical outcome. Other factors involved in EZH2 regulation, such as miR-101, should be investigated in accessible samples in order to improve the clinical management of advanced PDAC.

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KEYWORDS: EZH2 ■ gemcitabine-based chemotherapy ■ locally advanced/metastatic ■ outcome ■ pancreatic ductal adenocarcinoma ■ SNP

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Pancreatic ductal adenocarcinoma (PDAC) is a major unsolved health problem, with less than 5% of patients alive 5 years after diagnosis. Most patients (~80%) present with advanced disease (i.e., locally advanced or metastatic) at diagnosis. The primary goals of treatment in this setting are survival prolongation and palliation, but PDAC is notoriously resistant to systemic treatments [1].

Identification of key factors to select patients with the highest likelihood of responding, while minimizing useless and toxic treatments, is urgently needed. Several genetic alterations have been associated with PDAC aggressive behavior and chemoresistance [2], while epigenetic factors that play a role in tumor progression have recently been discovered. In this context, EZH2 is becoming increasingly acknowledged as a prognostic biomarker in radically resected PDAC patients [3].

EZH2 is a catalytic subunit of the Polycomb group (PcG). PcG proteins can repress gene expression by forming multiple complexes leading to histone methylation, thus resulting in epigenetic control of gene expression [4]. In particular, EZH2 can silence several tumor suppressor genes by trimethylating lysine 27 of histone H3, which plays a key role in tumor development [5]. Furthermore, EZH2 is crucial for cancer stem cell self-renewal in several cancer types [6], including PDAC, where EZH2 overexpression has been associated with decreased E-cadherin expression, invasion and poor prognosis [3,7]. EZH2 is also an important factor in PDAC cell chemoresistance, since EZH2 depletion by RNA interference or inhibition by DZNep sensitized PDAC cells to gemcitabine [7,8].

Therefore, our study aimed at evaluating the prognostic value of EZH2 expression in a subset of locally advanced or metastatic PDACs. Moreover, since blood samples are much more easier to obtain, especially for advanced cancers, and recent studies suggested a role for candidate polymorphisms of *EZH2* in lung cancer risk and colorectal cancer prognosis [9–11], we performed a pharmacogenetic study of two candidate *EZH2* polymorphisms.

The *EZH2* gene contains 20 exons, 19 introns [12] and 41 identified SNPs, and encodes two isoforms of different transcript sizes [9]. Among these SNPs, we selected rs6950683 and rs3757441 because individuals carrying C/C alleles at these two SNPs have a lower risk of lung cancer than those carrying the T/T wild-type allele [9]. rs3757441 is an intronic polymorphism, and might affect gene expression through several mechanisms, including changes in transcription factor binding sites, splicing variants and miRNA-targeting sequences. rs6950683, is located upstream of exon 1, and, therefore, may impact gene expression by affecting promoter function. Although the functional role of rs6950683 and rs3757441 has not yet been tested in experimental models

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[13], a recent study in 220 patients affected by hepatocellular carcinoma, and 552 cancer-free controls, demonstrated that individuals carrying at least one C allele at rs6950683 and rs3757441 had a significantly lower risk of developing hepatocellular carcinoma than wild-type (T/T) individuals [14].

Therefore we studied whether the *EZH2* rs6950683 and rs3757441 might influence EZH2 expression and predict outcome, using several cohorts of locally advanced or metastatic PDACs.

Materials & methods

Patients

EZH2 mRNA, protein levels and polymorphisms were evaluated initially in a cohort of 32 out of the 36 locally advanced or metastatic PDAC patients enrolled in a retrospective study on determinants of gemcitabine activity [15]. Patients' characteristics are described in Supplementary Table 1 (see www.futuremedicine.com/doi/suppl/10.2217/pgs.13.225).

RNA and DNA were extracted from laser microdissected biopsies, obtained before chemotherapy, using the LMD7000 instrument (Leica-Microsystems, Wetzlar, Germany), as described previously [16]. The laser microdissection procedure adopted in this study and the methods used to validate the purity of our PDAC samples are discussed in the Supplementary Material.

To validate the data of EZH2 expression from the first cohort, we selected a second cohort of 25 metastatic PDAC patients (Supplementary Table 1).

Further polymorphism analyses were performed in germline DNA extracted from blood samples of 340 patients, including 247 treated with the four-drug regimens cisplatin–docetaxel–capecitabine–gemcitabine (PDXG) and cisplatin–epirubicin–capecitabine–gemcitabine (PEXG or EC–GemCap), and 93 treated with gemcitabine (Supplementary Table 1). In these two larger cohorts we performed several stratified analyses of outcome according to chemotherapy regimens and clinical variables [17,18], but no tissues were available for studies on *EZH2* mRNA and protein levels.

All the eligible subjects were chemo-naive patients with cytologically or histologically proven stage III or IV PDAC, treated in four hospitals (Supplementary Table 2). The study was approved by the local ethics committees.

Analysis of mRNA/protein expression & genotypes

Genomic RNA and DNA were extracted from tissues and blood samples using the QIAmp® kits according to the manufacturer's protocol (Qiagen, CA, USA). *EZH2* mRNA values were evaluated by quantitative PCR, and expressed as arbitrary units (a.u.) normalized to β-actin [7]. *EZH2* rs3757441 and rs6950683 genotyping was performed with specific primers/probes (Supplementary Table 3) using the ABI-PRISM® (Applied Biosystems, Life Technologies, CA, USA) instrument for Taqman®-based PCR reactions (Applied Biosystems, Life Technologies), as described previously [17]. The concordance of these polymorphisms in tumor and germline DNA was studied in 25 paired samples of DNA extracted from PDAC samples and blood of the second cohort of patients, showing identical interindividual genotypes between normal and malignant tissues. No discrepancies were also observed in the samples analyzed in duplicate (~15%), and all the genotyping data were included in the final analysis.

The correlation between mRNA and protein expression was firstly evaluated in 20 selected cases of the first cohort of patients, where protein expression was studied using a specific antibody against EZH2 (Abcam-ab109398, 1:30 dilution; Abcam, MA, USA), with the avidin–biotin–peroxidase complex technique (Vectastain ABC kit; Vector Laboratories, CA, USA). Sections were reviewed by two researchers blinded to genetic data, who scored the immunostaining on the basis of staining intensities and number of stained cells (H-score), as described in Supplementary Table 4.

Then, we evaluated EZH2 protein expression in the second cohort, using a tissue microarray (TMA), with four different tumor areas for each patient, constructed with core tissue biopsies (diameter 1 mm) with the TMA Grand Master instrument (3DHistech, Budapest, Hungary).

Analysis of miRNA-101 expression

EZH2 expression can be regulated by mechanisms other than SNPs, and regulation of EZH2 by miR-101 has been already reported in PDAC [19]. Therefore, we performed a pilot study on the expression of this miRNA using the remaining RNA of 25 cases from the first cohort of PDAC patients, including the 20 cases with data on EZH2 protein expression.

RNA (10 ng) was reverse transcribed and the resulting cDNA was amplified using the specific TaqMan-MicroRNA-assays (Applied Biosystems, CA, USA) for miR-101 and RNU6 (Assay-ID 002253 and 001093, respectively). The PCR reactions were performed as described previously [20]. Specimens were amplified in

duplicate with appropriate nontemplate controls. Amplification data were normalized to RNU6 expression and quantification of relative expression (reported as a.u.) was performed using the ΔCt method.

Statistics

Demographic and clinical information was obtained from medical records. Overall survival (OS) and progression-free survival were analyzed from the day of treatment start to the end point (death/progression or censoring) according to the Kaplan–Meier method, and compared by log-rank test. Correlation with *EZH2* genotypes was evaluated grouping patients according to the three genotypes and collapsing homozygous and heterozygous genotypes when they had the same direction of effect, as described previously [18]. Data were analyzed using SPSS-20 software (IBM, IL, USA). All the analyses were two-sided and statistical significance was set at $p < 0.05$. A p-value between 0.05 to 0.10 was considered as a trend toward significance.

Results

EZH2 mRNA correlates with survival, rs6950683 polymorphism & protein expression

EZH2 mRNA expression was detectable in all microdissected samples. The RNA yield ranged between 27–112 μg, and 250 ng were used for cDNA synthesis, obtaining Ct values between 26 and 30 in the Taqman-based PCR reactions. Moreover, quantitative PCR data showed a

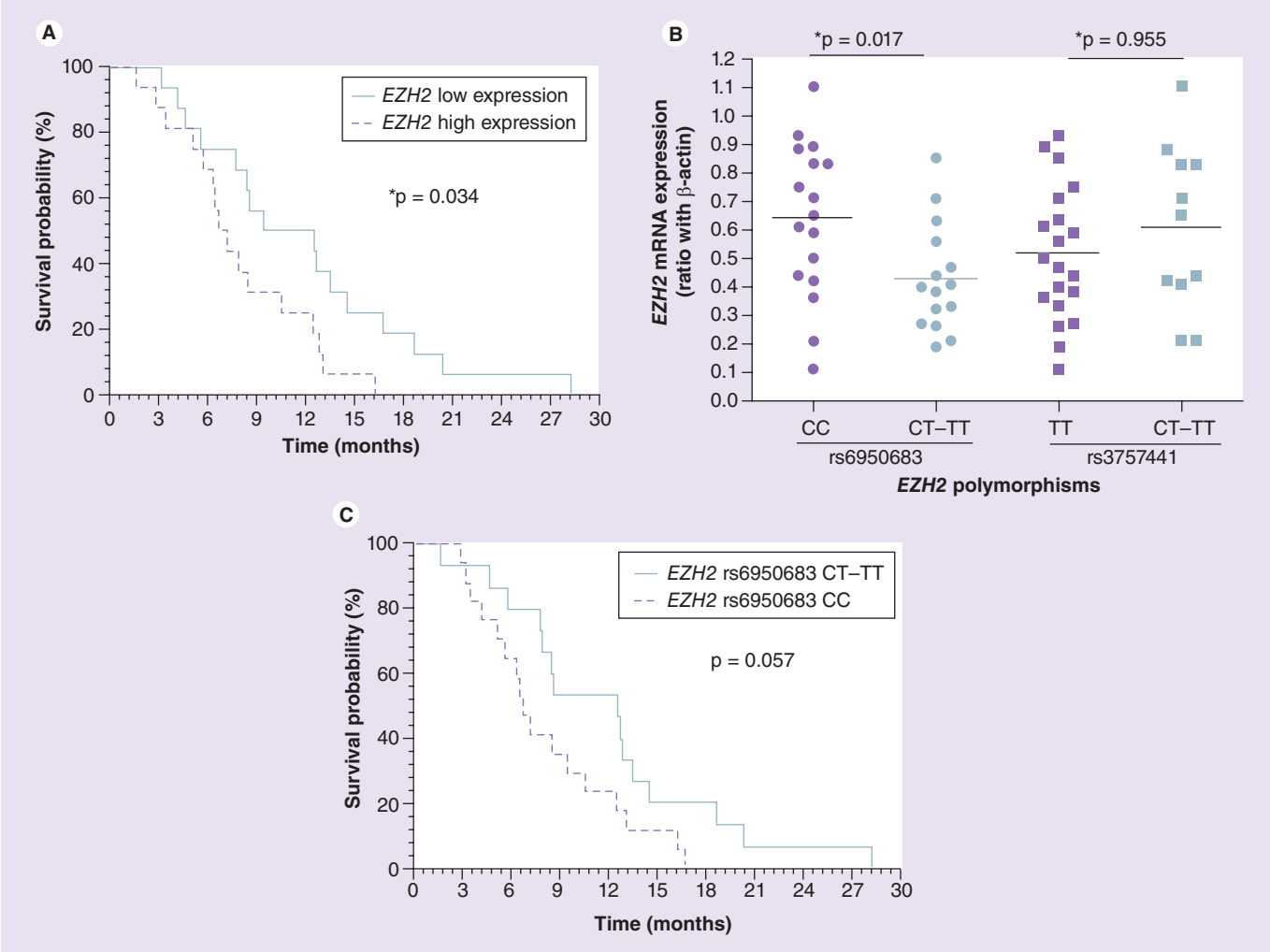

Figure 1. *EZH2* mRNA expression and polymorphisms in the first cohort of patients. (A) Kaplan–Meier survival curves according to *EZH2* mRNA expression genotypes in the first cohort of pancreatic ductal adenocarcinoma patients (n = 32). The *EZH2* low expression group had a median *EZH2* mRNA expression of 0.35 a.u. (range: 0.11–0.47), while the *EZH2* high expression group had a median *EZH2* mRNA expression of 0.73 a.u. (range: 0.50–1.10). **(B)** *EZH2* mRNA expression levels according to the *EZH2* rs3757441 and rs6950683 polymorphisms. **(C)** Kaplan–Meier survival curves according to *EZH2* rs6950683 polymorphism.
*p < 0.05.

variability coefficient of Ct always lower than 2% of mean values.

Patients were categorized into two subgroups with respect to the median mRNA expression and evaluated for clinical outcome after gemcitabine chemotherapy (Figure 1A). The high *EZH2* expression group had a significantly poorer prognosis (median OS of 6.7 months, 95% CI: 5.3–8.0, compared with 9.4 months, 95% CI: 1.6–17.2, in patients with low expression levels). Similar results were observed for progression-free survival, with median values of 4.4, 95% CI: 3.2–5.2, versus 8.4 months, 95% CI: 5.5–11.3 (p = 0.04), in patients with high and low *EZH2* expression, respectively.

Remarkably, EZH2 expression profile was lower in grade 1/2 (n = 13) than grade 3 (n = 19) tumors (0.39 ± 0.16 vs 0.64 ± 0.26 a.u., respectively, p < 0.05 in the Wilcoxon rank-sum test). By contrast, no difference was detected according to other clinicopathological parameters (Table 1).

The plot in Figure 1B shows the variability of gene expression across the cohort of 32 patients subjected to transcription analysis according to *EZH2* polymorphisms. The rs6950683 C/C genotype was associated with a significantly higher *EZH2* expression, and patients carrying this genotype (n = 17) had a OS of 6.7 months, 95% CI: 5.5–7.9, versus 12.5 months, 95% CI: 7.1–17.9 in patients (n = 15) harboring the C/T–T/T genotypes (Figure 1C). Notably patients with the C/T genotype (n = 13) had an intermediate OS (12.5 months, 95% CI: 3.0–13.9), but no further statistical analysis was performed on these subgroups because of the lack of power caused by the low number of cases. Similarly, we did not perform multivariate analyses or additional stratified analyses according to clinical variables.

Immunohistochemistry revealed variable protein expression related to mRNA expression (Figure 2A). Indeed, the tissues characterized by high *EZH2* mRNA expression, presented a strong and diffuse staining of the EZH2 protein (Figure 2A, upper panel), while the tissues with low *EZH2* expression had only a few scattered positive cells with a weak nuclear staining (Figure 2A, lower panel). However, higher EZH2 protein expression was associated with the rs6950683 C/C SNP (Figure 2B) and correlated with shorter OS (Figure 2C). Patients with high EZH2 protein expression had a significantly shorter median OS compared with patients with low EZH2 protein expression (6.9 months, 95% CI: 5.4–14.3 vs 12.7 months, 95% CI: 9.3–14.4, p = 0.040).

Additional analysis of EZH2 protein expression was performed on tumor tissues from 25 metastatic PDAC patients collected in a TMA (Figure 3A), this showed that low EZH2 protein expression correlated with longer OS (10.4 months, 95% CI: 7.4–13.6 vs 3.0 months, 95% CI: 2.5–3.5, p = 0.022, Figure 3A).

The rs6950683 C/C SNP was significantly associated with higher EZH2 protein expression (p = 0.003, Figure 3B). Patients carrying this genotype (n = 15) had a median OS of 4.0 months, 95% CI: 2.2–8.4, compared with 9.4 months, 95% CI: 1.9–16.9 for patients (n = 10) harboring the C/T–T/T genotypes (p = 0.063, Figure 3C).

Table 1. Association of EZH2 expression with clinicopathological covariates in the first cohort of pancreatic ductal adenocarcinoma patients.

Covariate	*EZH2* mRNA expression		Total, n (%)	p-value
	Low, n (%)	*High, n (%)*		
Sex				
Male	10 (62.5)	12 (75.0)	22 (68.8)	0.704
Female	6 (37.5)	4 (25.0)	10 (31.2)	
Age				
≤65 years	9 (56.3)	11 (68.8)	20 (62.5)	0.716
>65 years	7 (43.7)	5 (31.2)	12 (37.5)	
PS				
≤80	2 (12.5)	4 (25.0)	6 (18.8)	0.654
>80	14 (87.5)	12 (75.0)	26 (82.2)	
Stage				
III	4 (25.0)	1 (6.3)	5 (15.6)	0.333
IV	12 (75.0)	15 (93.7)	27 (84.4)	
Grading (WHO)				
G1–G2	10 (62.5)	3 (18.7)	13 (40.6)	0.029
G3	6 (77.5)	13 (81.3)	19 (59.4)	

G: Grade; PS: Performance status.

EZH2 polymorphisms & outcome in the validation cohorts

The shorter median OS observed in patients with the rs6950683 C/C genotype in our first two cohorts (with p-values indicating a trend toward a significant difference), as well as the possible correlation of rs6950683 with EZH2 expression, prompted additional pharmacogenetic studies in two larger 'validation' cohorts (Table 2). Genotyping was successfully carried out in all these samples. Although the purpose of this analysis was not to perform a case–control study, we compared the baseline demographic characteristics, and in the first cohort of patients treated with gemcitabine monotherapy we

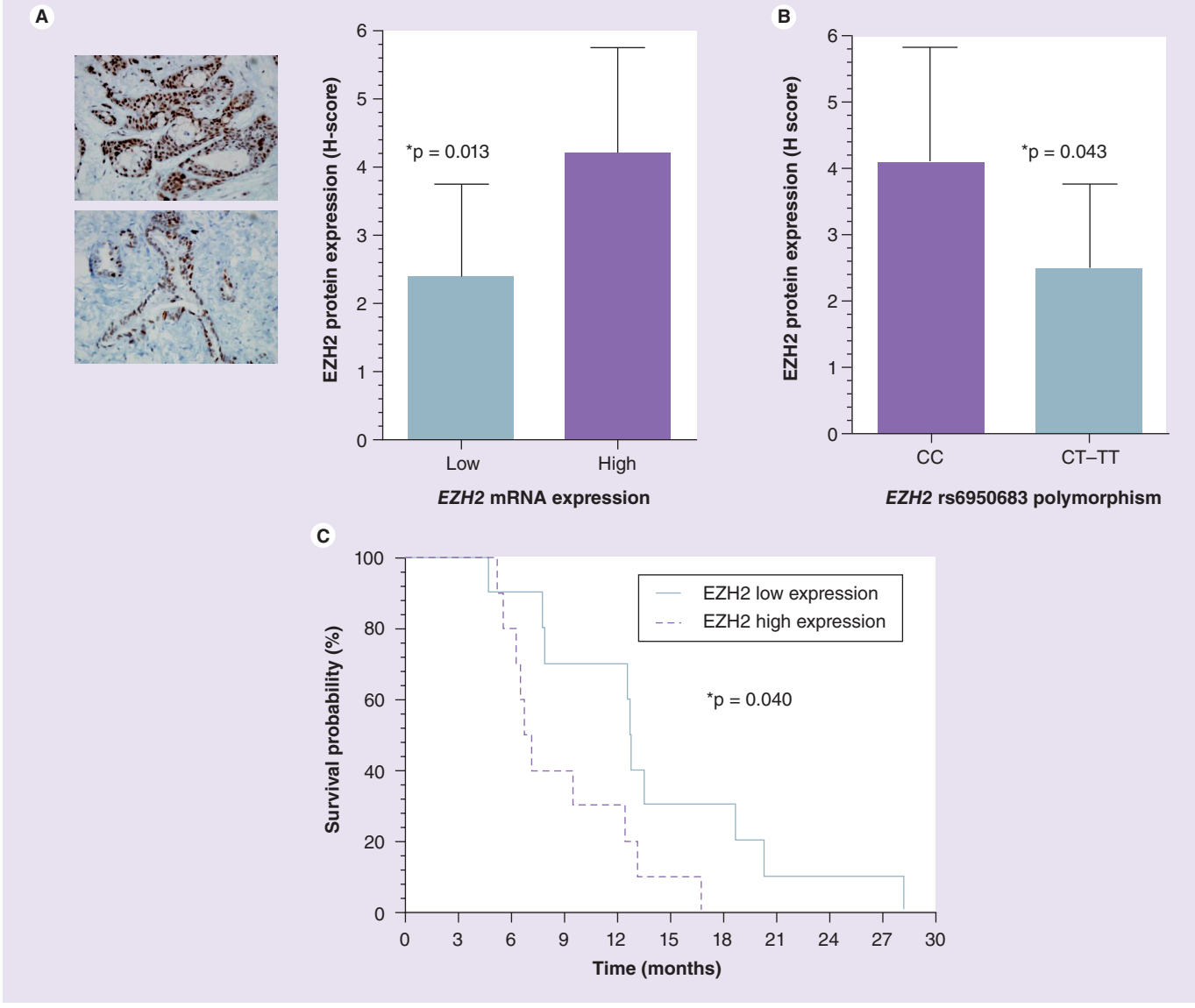

Figure 2. EZH2 protein expression and polymorphisms in the first cohort of patients. (A) Correlation of *EZH2* mRNA and protein expression, as assessed by PCR and immunohistochemistry (see representative immunohistochemistry), respectively. Columns: mean values; bars: standard deviation. **(B)** EZH2 protein expression levels according to the *EZH2* rs6950683 polymorphism (n = 20). Columns: mean values; bars: standard deviation. **(C)** Kaplan–Meier survival curves according to EZH2 protein expression levels.
*p < 0.05.

observed a significantly higher percentage of old patients (age >65 years, p = 0.008), but no significant differences were found for gender, performance status, stage or allelic frequencies across the series (Supplementary Table 1). However, patients treated with the polychemotherapeutic regimens had significantly longer OS, as reported previously [18].

No correlations were detected between genotypes and clinicopathological parameters (data not shown). Similarly, no significant differences were observed in OS for *EZH2* polymorphisms in the gemcitabine monotherapy and PDXG, PEXG and EC–GemCap cohorts (Table 2).

miR-101 expression correlates with EZH2 protein expression & clinical outcome

miR-101 binds the *EZH2* 3′-UTR at two sites (Figure 4A), and was recently shown to interact with EZH2 in several types of cancer [21]. Therefore, we evaluated miR-101 expression levels in 25 cases of the first cohort of PDAC patients. These patients were categorized according to median expression value, according to the Gaussian distribution of the expression values of this miRNA (data not shown). Remarkably, we observed a significant inverse relationship (p = 0.004) between the

expression of miR-101 and the expression of EZH2 (Figure 4B).

Moreover, a strong interaction of miR-101 expression status and clinical outcome was demonstrated. The low miR-101 expression group had a poorer prognosis than the high expression group. Patients (n = 13) with miR-101 expression above the median (high miR-101) had a significantly longer median OS (12.7 months, 95% CI: 10.3–15.2) compared with patients (n = 12) with miR-101 expression lower than the median (low miR-101, 7.2 months, 95% CI: 4.2–10.2, p = 0.013). The OS Kaplan–Meier curves are shown in Figure 4C.

Discussion

This is the first study supporting a role for *EZH2* as a novel prognostic factor in advanced PDAC patients treated with gemcitabine. Moreover we demonstrated that *EZH2* mRNA expression correlated with protein expression and tumor grading.

Previous findings reported that nuclear accumulation of EZH2 was associated with increased invasiveness and poor prognosis in radically resected patients [3]. Several studies showed that more advanced/malignant pancreatic tumors expressed higher levels of EZH2 [22]. In an analysis of the Oncomine database, two datasets on

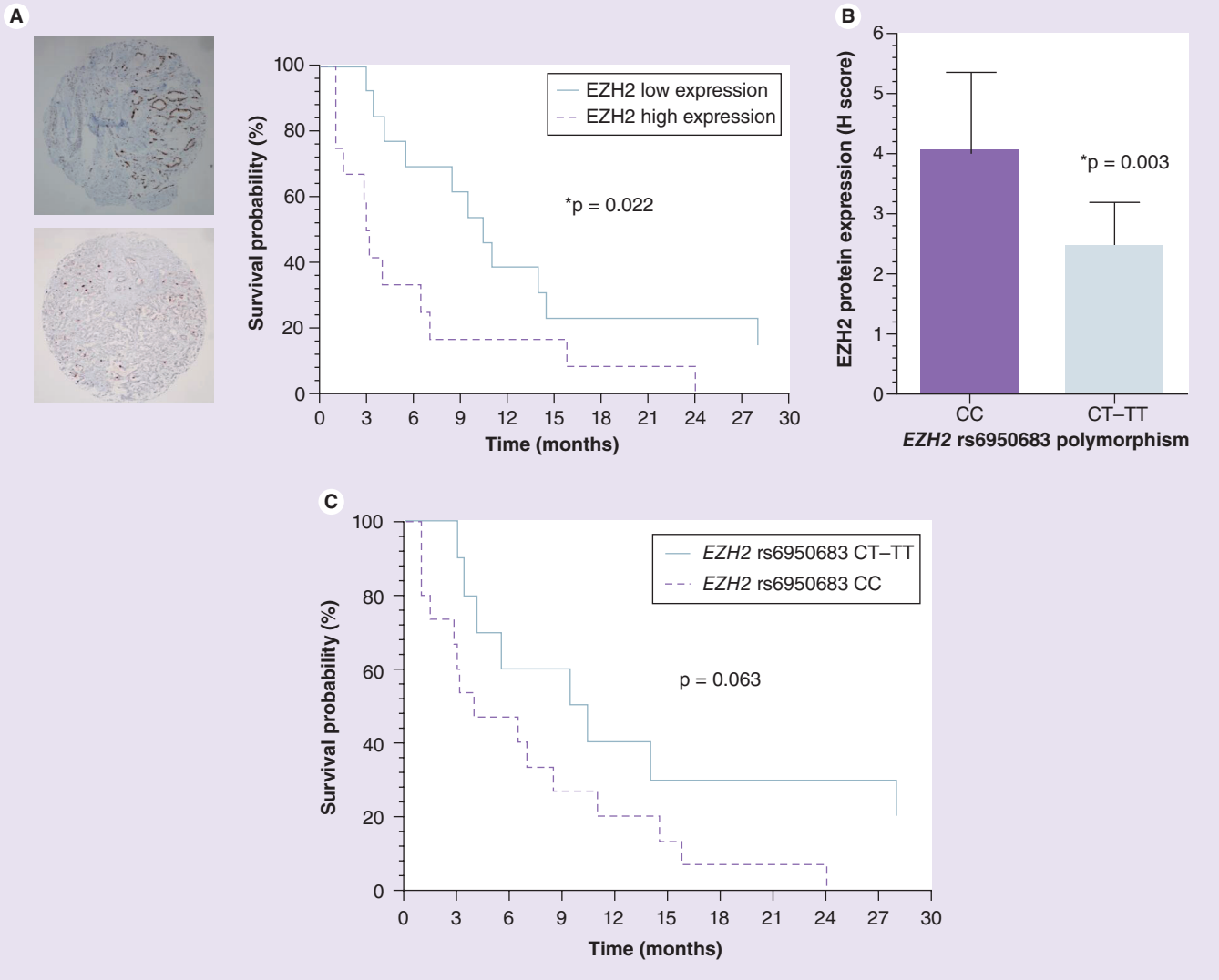

Figure 3. EZH2 protein expression and polymorphisms in the second cohort of patients. (A) Kaplan–Meier survival curves according to EZH2 protein expression levels. EZH2 protein expression assessed by immunohistochemistry in tissue microarray (see representative cores for high and low expression, in the upper and lower panel, respectively), including the tissues from the second cohort of pancreatic ductal adenocarcinoma patients (n = 25). **(B)** EZH2 protein expression levels according to the *EZH2* rs6950683 polymorphism. Columns: mean values; bars: standard deviation. **(C)** Kaplan–Meier survival curves according to *EZH2* rs6950683 polymorphism.
*p < 0.05.

Table 2. Clinical outcome according to polymorphisms in the validation cohorts.

SNP	Genotype	Patients, n (%)	HWE p-value	OS mo. (95% CI)	p-value	PFS mo. (95% CI)	p-value
Gemcitabine monotherapy cohort							
rs3757441	TT	61 (65.6)	0.211	7.8 (5.8–9.8)	0.494†	6.3 (5.2–7.3)	0.269†
	CT	26 (28.0)		6.8 (4.3–9.2)		5.6 (2.8–8.3)	
	CC	6 (6.4)		6.8 (2.6–13.1)		2.8 (2.6–7.3)	
	CT+CC	32 (34.4)		6.9 (5.0–8.7)	0.498‡	5.6 (3.2–8.0)	0.416‡
rs6950683	CC	65 (69.9)	0.180	7.9 (5.9–9.9)	0.342†	6.3 (4.8–7.7)	0.331†
	CT	23 (24.7)		7.0 (6.0–8.0)		6.0 (4.5–7.7)	
	TT	5 (5.4)		6.6 (2.8–10.4)		5.0 (2.6–7.4)	
	CT+TT	28 (30.1)		7.0 (6.2–7.9)	0.269‡	6.0 (4.8–7.2)	0.162‡
PDXG, PEXG & EC–GemCap cohort							
rs3757441	TT	153 (61.9)	0.126	12.0 (10.7–13.3)	0.470†	10.0 (9.0–11.0)	0.187†
	CT	73 (29.6)		12.0 (10.4–13.6)		8.0 (6.6–9.3)	
	CC	21 (8.5)		12.0 (7.8–16.2)		7.0 (5.9–8.0)	
	CT+CC	94 (38.1)		12.0 (10.4–13.6)	0.494‡	8.0 (6.3–9.7)	0.873‡
rs6950683	CC	145 (58.7)	0.484	12.0 (10.7–13.3)	0.676†	7.3 (4.2–1–.4)	0.110†
	CT	93 (37.7)		12.0 (9.8–14.2)		8.6 (7.6–9.5)	
	TT	9 (3.6)		12.0 (8.6–15.4)		10.2 (7.9–12.4)	
	CT+TT	102 (41.3)		12.0 (10.1–14.4)	0.468‡	10.0 (7.7–12.3)	0.282‡

†p-values were calculated with Log-rank test grouping patients according to the three genotypes.
‡p-values were calculated with Log-rank test collapsing polymorphic homozygous and heterozygous genotypes, as described previously [18].
HWE: Hardy–Weinberg equilibrium; mo.: Months; OS: Overall survival; PDXG: Cisplatin–docetaxel–capecitabine–gemcitabine; PEXG/EC–GemCap: Cisplatin–epirubicin–capecitabine–gemcitabine; PFS: Progression-free survival.

PDAC showed significantly increased levels of EZH2 in PDAC compared with normal pancreas tissues, as reported in Supplementary Figure 1 [23]. However, in 38 endoscopic ultrasound/fine-needle aspiration samples the staining intensity of EZH2 did not differ in moderately and poorly differentiated PDAC samples [24]. These controversial data might be explained by technical issues such as limited sampling and the heterogeneity of PDAC specimens that we enriched for tumor cell content by laser microdissection. Still, more studies are difficult to perform because of the very small amount of tissue available from most advanced PDAC patients. Therefore we evaluated candidate polymorphisms, and found that the C/T and T/T variants of rs6950683 were associated with a lower expression of *EZH2*. These results might explain the protective role of these genotypes in lung and hepatocellular cancers [9,14]. Conversely, no correlation was observed with the rs3757441 polymorphism, which was previously associated with outcome in colorectal cancer patients treated with first-line 5-fluorouracil, folinic acid, irinotecan (FOLFIRI) with or without bevacizumab [10–11], suggesting that its prognostic role might be tumor specific.

Encouraged by the discovery of a trend toward a significant association of the rs6950683 polymorphism with survival in the first two cohorts of gemcitabine-treated patients, we tested its clinical impact in two larger homogeneous cohorts. These cohorts included patients treated with gemcitabine alone and with polychemotherapeutic regimens from a multicentric series, to evaluate possible treatment-related effects.

Unfortunately, no correlation with outcome was observed in both cohorts. This lack of correlation might be explained by several factors, including first of all the small sample size of the first two cohorts. However, no data was available on EZH2 expression in the two larger cohorts, and in these cohorts we could not evaluate the association between our candidate SNPs and expression, or expression with survival.

Our candidate SNPs were selected according to previous studies, but they are in linkage disequilibrium with other *EZH2* polymorphisms [9], which might play a more determinant role. Although *EZH2* mutations have not yet been reported in PDAC, we cannot exclude that such somatic alterations might occur [22], affecting EZH2 expression and function in tumor tissues,

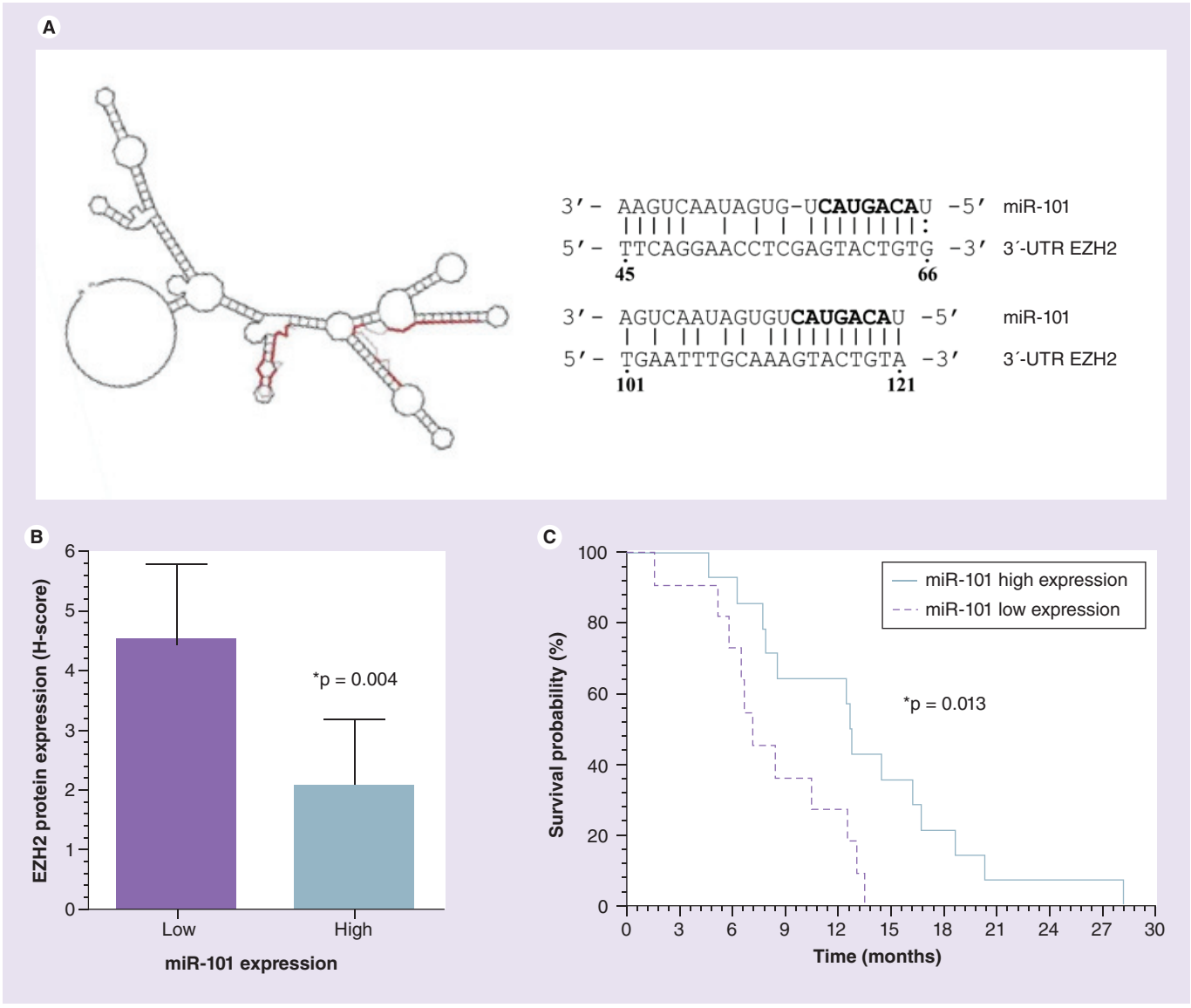

Figure 4. Correlation of miR-101 with EZH2 protein expression and survival in the first cohort of patients. (A) Predicted RNA structure of the 3′-UTR of EZH2 by RNAfold software, as described previously [21,25], in red are indicated the two miR-101 binding sites. **(B)** EZH2 protein expression levels according to miR-101 expression levels. Columns: mean values; bars: standard deviation. **(C)** Kaplan–Meier survival curves according to miR-101 expression.
*$p < 0.05$.
For color images please see www.futuremedicine.com/doi/pdf/10.2217/pgs.13.225.

regardless of its genotype. A recent study demonstrated the pivotal role of EZH2 during the suppression of miR-218 and revealed a new silencing mechanism involving EZH2-induced *de novo* heterochromatinization in PDAC biology [26]. This heterochromatin induction at the miR-218 promoter adds an extra dimension to EZH2-mediated aberrant epigenetic effects in PDAC, supporting further analyses on functional targets of miR-218, such as UGT8 and VOPP1, to dissect the role of EZH2. Similarly, several previous studies suggested the key role of miR-101 in the regulation of EZH2 expression [19–21]. This was confirmed in our pilot analysis in PDAC tissues of the first cohort of patients, and should prompt future studies on miRNAs affecting EZH2 expression in larger cohorts of patients.

A major strength of the present study is that it was performed on several cohorts of PDAC patients, including patients from multicenter studies, who were all treated with upfront chemotherapy. Conversely, the main limitations of this study include the retrospective explorative study design, the modest sample size and lack of multivariate analysis of the first two cohorts, and the possible confounding influence of drug combinations on the treatment–SNP effects. Recent preclinical data supported a key role of EZH2 in the

sensitivity of PDAC cells to gemcitabine [7], but several studies reported a general prognostic role of EZH2, independent from the therapeutic strategy [22]. The planning of randomized studies with a control arm of patients treated with other regimens, such as 5-fluorouracil/leucovorin combined with irinotecan and oxaliplatin (FOLFIRINOX), and the comparison of the survival stratified by genotype would be the only way to establish the specific predictive role of EZH2 for the activity of gemcitabine.

Conclusion & future perspective

EZH2 expression emerged as a prognostic factor for locally advanced/metastatic PDAC, but candidate polymorphisms could not predict outcome. Other factors involved in the EZH2 oncogenic pathways or chemoresistance and detectable in accessible samples sources, such as miRNA enriched tumor-derived exosomes in peripheral blood [27], should be investigated in order to improve the clinical management of advanced PDAC patients.

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Background

- Pancreatic ductal adenocarcinoma (PDAC) is a major unsolved health problem. Most patients present with advanced disease at diagnosis, and identification of key factors that play a critical role in chemoresistance should offer room for treatment optimization.
- EZH2 is becoming increasingly acknowledged as a prognostic biomarker in radically resected PDAC patients, and EZH2 is also an important factor in PDAC cell chemoresistance to gemcitabine.
- SNPs in *EZH2* have been correlated with lung cancer risk and colorectal cancer prognosis, possibly affecting EZH2 expression.
- The aim of the current study was to evaluate the correlation between these candidate SNPs and EZH2 expression, as well as with clinical outcome in four cohorts of PDAC patients treated with gemcitabine or with polychemotherapeutic regimens.

Pharmacogenetics of* EZH2 *in PDAC patients

- In a cohort of PDAC laser microdissected specimens, *EZH2* mRNA levels correlated with survival: the high EZH2 expression group had a significantly poorer prognosis. Similar results were observed for progression-free survival.
- Immunohistochemistry revealed a correlation between *EZH2* mRNA and protein expression in resected PDACs, and EZH2 expression correlated with overall survival (OS) in two cohorts of locally advanced/metastatic patients treated with gemcitabine.
- In these cohorts, the rs6950683 C/C genotype was associated with a significantly higher EZH2 expression, and patients carrying this genotype had a trend towards a significantly shorter OS.
- However, no significant differences were observed in OS for *EZH2* polymorphisms in two larger cohorts of patients treated with gemcitabine and with polychemotherapeutic regimens.

Conclusion & future perspective

- The results of the present study demonstrated the prognostic role of EZH2 in advanced PDAC patients treated with gemcitabine. Moreover we demonstrated that *EZH2* mRNA expression correlated with protein expression and tumor grading.
- Although the rs6950683 C/C genotype correlated with EZH2 expression, no correlation with outcome was observed in two validation cohorts. This lack of correlation might be explained by several factors including the modulation of EZH2 expression by miRNA-101, which was observed in the first cohort of patients.
- Since PDAC is such a dismal disease, any biomarker that can help to better stratify patients might have crucial clinical applications.
- Other factors involved in the regulation of EZH2 expression/activity and detectable in accessible samples sources should be investigated in order to improve the clinical management of advanced PDAC patients.

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