Loss of the CBX7 protein expression correlates with a more aggressive phenotype in pancreatic cancer

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ABSTRACT

Polycomb group (PcG) proteins function as multiprotein complexes and are part of a gene regulatory mechanism that determines cell fate during normal and pathogenic development. Several studies have implicated the deregulation of different PcG proteins in neoplastic progression.

Pancreatic ductal adenocarcinoma is an aggressive neoplasm that follows a multistep model of progression through precursor lesions called pancreatic intraepithelial neoplasia (PanIN).

Aim of this study was to investigate the role of PcG protein CBX7 in pancreatic carcinogenesis and to evaluate its possible diagnostic and prognostic significance.

We analysed by immunohistochemistry the expression of CBX7 in 210 ductal pancreatic adenocarcinomas from resection specimens, combined on a tissue microarray (TMA) including additional 40 PanIN cases and 40 normal controls. The results were evaluated by using receiver operating characteristic (ROC) curve analysis for the selection of cut off scores and correlated to the clinicopathological parameters of the tumours and the outcome of the patients. Expression of E cadherin, a protein positively regulated by CBX7, was also assessed.

A significantly differential, and progressively decreasing CBX7 protein expression was found between normal pancreatic tissue, PanINs and invasive ductal adenocarcinoma. Loss of CBX7 expression was associated with increasing malignancy grade in pancreatic adenocarcinoma, whereas the maintenance of CBX7 expression showed a trend toward a longer survival. Moreover, loss of E cadherin expression was associated with loss of CBX7 and with a trend towards worse patient survival.

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These results suggest that CBX7 plays a role in pancreatic carcinogenesis and that its loss of expression correlates to a more aggressive phenotype.

1. Introduction

Polycomb group proteins (PcGs) appear to function as multi protein complexes and are part of a gene regulatory mechanism that determines cell fate during normal and pathogenic development.¹ Biochemical and genetic studies indicate that PcG proteins act as part of at least two high molecular weight complexes: Polycomb repressive complexes 1 and 2 (PRC1 and PRC2).² The components of the PRC1 complex are the mammalian homologues of *Drosophila* Polycomb (Pc), Posterior sex combs (Pscs), Sex combs extra (Sce) and Polyhomeiotic (Ph). CBX7 is a Pc homologue consisting of a conserved chromodomain near the aminoterminal and a Polycomb box in the carboxy terminal region.

Several studies have implicated the deregulation of different PcG proteins in tumorigenesis.³⁻⁵ We have recently shown that in thyroid neoplasia CBX7 expression progressively decreased with malignancy grade and neoplasia stage.⁶ Indeed, CBX7 decreased in a growing number of cases going from benign adenomas to papillary, follicular and anaplastic thyroid carcinomas. Equally, a correlation between loss of CBX7 expression and a poor survival was found in human colon and breast carcinomas (Dr. Pallante P. NOGEC, Naples, Italy). Moreover, restoration of CBX7 expression in thyroid cancer cells reduced growth rate, with retention in the G1 phase of the cell cycle, suggesting a critical role of CBX7 in the regulation of transformed thyroid cell proliferation.⁶ More recently, we have demonstrated that CBX7 is able to positively regulate E cadherin expression by interacting with Histone deacetylase 2 and inhibiting its activity on the E cadherin promoter thereby accounting for the correlation between the loss of CBX7 expression and a highly malignant phenotype.⁷

Pancreatic ductal adenocarcinoma is the fourth leading cause of cancer death and has dismal prognosis.⁸ Clinicopathological parameters like tumour size, clinical stage, nodal metastases and evidence of blood vessel or lymphatic invasion have been proven to be reliable prognostic determinants in pancreatic cancer.^{9,10} However, earlier detection would help to improve patient outcome. Moreover, the identification of reliable and reproducible prognostic markers would enable the stratification of patients into different groups, and would eventually provide a guide in developing new therapeutic modalities. It is known that pancreatic cancer follows a multistep model of progression through non invasive precursor lesions.^{11,12} Recently, pancreatic intraductal lesions have been classified into four groups of pancreatic intraepithelial neoplasias (PanINs): PanIN 1A, 1B, 2 and 3.^{12,13} PanIN 3 demonstrates severe epithelial dysplasia and is most likely to progress to invasive carcinoma.¹⁴

The objective of the present study was to investigate the role of the PcG protein CBX7 in pancreatic carcinogenesis and to evaluate its diagnostic and prognostic significance. We therefore analysed immunohistochemically possible

differences in the CBX7 expression between invasive adenocarcinomas of the pancreas, PanINs and normal pancreatic tissue, in 210 ductal adenocarcinomas of the pancreas from resection specimens, combined on a tissue microarray (TMA) including 40 PanIN 3 cases and 40 normal controls. E cadherin expression was additionally assessed.

The results were evaluated by using receiver operating characteristic (ROC) curve analysis for the selection of cut off scores,^{15,16} and correlated to the clinicopathological parameters of the tumours.

2. Material and methods

2.1. Patients and specimens

Formalin fixed and paraffin embedded tumours and control specimens were retrieved from the archives of the Institute of Pathology, University of Bern. All tumours and controls were reviewed by an experienced pathologist (EK). Histologies other than ductal carcinoma were excluded. Tumours were restaged according to the American Joint Committee on Cancer (AJCC) Staging Manual (sixth edition). Representative tumour areas were selected for the construction of the tissue microarray (TMA). The TMA consisted of 210 cases of ductal adenocarcinoma of the pancreas including 40 PanIN 3 cases and 40 normal controls (normal pancreatic tissue and PanINs were selected from areas distant to the carcinomas). The 210 patients comprised 110 males and 100 females with a mean age of 66.5 years (range 20-92).

The study was approved by the ethical committee of the University of Bern.

2.2. Assessment of behaviour

Medical charts were available from 77 of 210 patients. Of these 77 patients, 60 (78%) died of the disease, and 7 (9%) were alive with recurrent/metastatic disease. Additional 10 patients (13%) were alive without disease. The median follow up was 16 months. The characteristics of carcinomas with survival information are shown in Table 1.

2.3. Construction of tissue microarray

TMA was built as previously described.¹⁷ Briefly, one core tissue biopsy with a diameter of 0.6 mm was taken from a representative region of individual paraffin embedded pancreatic carcinomas (donor blocks) and was placed into a new recipient paraffin block using a semiautomated tissue arraying device. The presence of tumour tissue on the TMA was verified on a haematoxylin eosin stained slide. Two to three tissue cores of each tumour were available for biomarker analysis. About 5 µm sections were cut using an adhesive coated slide system (Instrumedics Inc., Hackensack, NJ, United States of

Table 1 – Characteristics of carcinomas with survival information (N = 77).

Clinico pathological features		Frequency N (%)
Diagnosis	Ductal carcinoma	77 (100.0)
Sex	Female	33 (42.9)
	Male	44 (57.1)
Tumour grade	G1	16 (20.8)
	G2	42 (54.6)
	G3	19 (24.7)
pT stage	pT1	3 (4.1)
	pT2	12 (16.2)
	pT3	52 (70.3)
	pT4	7 (9.5)
pN stage	pN0	27 (38.0)
	pN1	43 (60.6)
	pN2	1 (1.4)
Metastasis	Absence	72 (93.5)
	Presence	5 (6.5)
Tumour diameter	Mean $\pm$ SD	3.14 $\pm$ 1.4
Survival time (months)	Median (range)	12.0 (0.5–48.0)

America) and examined by immunohistochemistry. The number of samples varied slightly between the individual markers because of variability in the number of interpretable specimens on TMA sections.

2.4. Immunohistochemistry

Freshly cut sections of TMA blocks were used for immunohistochemical staining with CBX7. Briefly, punches were dewaxed and rehydrated in dH₂O. Endogenous peroxidase activity was blocked using 0.5% H₂O₂. The sections were incubated with 10% normal goat serum (Dako Cytomation, Carpinteria, CA, USA) for 20 min and followed by the primary antibody at room temperature. Optimal staining could be achieved after pre-treatment with microwave oven (98 °C 30 min. pH6, dilution 1/250). Subsequently, sections were incubated with peroxidase labelled secondary antibody (DakoCytomation) for 30 min at room temperature. DAB was used as a chromagen. Sections were then counterstained with Gill's haematoxylin.

The CBX7 antibody used in this study was raised against the synthetic peptide C 18 R (TVTFREAQAAEGFFRDR) specific for the carboxy terminal (C terminal) region of the CBX7 protein. They were affinity purified against the synthetic peptide. Negative controls were performed by omitting the first antibody. The specificity of the reaction was confirmed by the lack of tissue immunoreactivity after pre incubation of the antibody with molar excess of the CBX7 synthetic peptide. For E cadherin staining a mouse monoclonal antibody (DakoCytomation, clone NCH 38) at a dilution 1:20 was used.

As positive control a tissue microarray with various normal tissue samples was stained in parallel.

2.5. Immunohistochemical evaluation

Nuclear CBX7 and membranous E cadherin staining were scored by two independent observers (L.T., E.K.) blinded for

clinical parameters. Slides were screened semi quantitatively for the percentage of positivity and the intensity of the signal. At least 100 cells were counted for each punch. The percentage of positive cells per number of cells counted was assessed in 10 groups from 0 (0–9%) to 9 (91–100%). Intensity of the signal was graded semi quantitatively in 4 groups from 0 (no positivity) to 3 (strong positivity). A case was considered positive if belonging at least to group 1 for the percentage (i.e. $\geq 10\%$) irrespective of intensity. In PanINs and normal controls the epithelial cells of ductal structures were evaluated.

2.6. Statistical methods

2.6.1. Selection of cut-off scores

The selection of clinically important cut off scores was based on ROC curve analysis.^{18,19} At each percentage score, the sensitivity and specificity for each outcome under study were plotted, thus generating a ROC curve. The score having the closest distance to the point with both maximum sensitivity and specificity, i.e. point (0.0, 1.0) on the curve, was selected as the cut off score leading to the greatest number of tumours which were correctly classified as having or not the outcome. In order to use ROC curve analysis, the clinico pathological features were dichotomised: T stage (early T1 + T2) or late (T3 + T4), N stage (N0; no lymph node involvement) or N1 (any lymph node involvement), tumour grade (low G1 + G2) or high (G3), and survival (death due to pancreatic carcinoma or alive).

Chi square tests were used to study the relationship between CBX7 and E cadherin expression and histological subgroups. Differences in CBX7 and E cadherin expression between normal tissue, PanIN and carcinoma were investigated using the non parametric Wilcoxon Rank Sum Test. Univariate survival analysis was carried out by the Kaplan Meier log rank test and multivariable analysis by Cox proportional hazards regression. Hazard ratios (HR) and 95% confidence intervals (CIs) were used to determine the effect of each variable on survival time. In addition, logistic regression was performed in univariate and multivariable settings to determine the association of CBX7 expression and its independent effect on binary outcomes. The odds ratios (ORs) and 95% CI were evaluated. A Bonferroni correction for multiple comparisons was performed. $P \leq 0.01$ (2 sided) were required for the association to be statistically significant. All analyses were carried out using SAS (V9, The SAS Institute, NC, USA).

3. Results

3.1. Analysis of CBX7 and E-cadherin expression by immunohistochemistry

A TMA consisting of 210 cases of ductal pancreatic carcinoma, 40 PanIN 3 cases and 40 normal pancreatic tissues was analysed by immunohistochemistry for CBX7 protein expression using polyclonal antibodies raised versus the carboxy terminal region of human CBX7 protein (see Methods) and E cadherin expression using a mouse monoclonal antibody. The clinico pathological features of the patients are reported in Table 1. Table 2 shows the correlation between CBX7 and E cadherin expression. The immunohistochemical findings are summarised in Tables 3–5. Some representative results

Table 2 – Association of E-cadherin and CBX7.

CBX7	E cadherin Freq (%)		P value
	Negative	Positive	
Negative	81 (90.0)	42 (39.6)	<0.001
Positive	9 (10.0)	64 (60.4)	
Total number of cases	90	106	

Table 3 – Mean expression of CBX7 from normal to PanIN to pancreas cancer (% of CBX7-positive cells).

Normal N 37	PanIN N 38	Cancer N 208
56.1%	36.6%	33.8%
Differences between normal and (PanIN + Cancer) $P < 0.0001$.		

of the immunohistochemical analysis are shown in Figs. 1 and 2.

3.2. Pancreatic carcinomas versus normal controls

In comparison with pancreatic carcinoma cases, normal tissue expressed significantly more frequently nuclear CBX7. In more detail, the median and mean protein expression for CBX7 was found to be 60% and 56.1%, respectively (% of CBX7 positive cells) in normal tissue, compared to 30% and 33.8%, respectively, in carcinomas ($P < 0.0001$, Table 3). Membranous E cadherin expression was also significantly more frequent in normal tissue (median and mean expression 30% and 33.9%, respectively), compared to carcinomas (median and mean expression 10% and 15.6%, respectively) ($P < 0.001$, Table 4).

3.3. Pancreatic carcinoma versus PanIN

Mean protein expression of CBX7 was higher in PanIN cases (median and mean expression 40% and 36.6%, respectively),

compared to pancreatic carcinomas (Table 3). Likewise, mean E cadherin expression was higher in PanINs (median and mean expression 20% and 30.9%, respectively) compared to carcinomas (Table 4).

3.4. PanIN versus normal controls

Mean protein expression of CBX7 was found to be significantly higher in normal tissue compared to PanIN cases ($P = 0.0001$, Table 3). Mean E cadherin expression was also higher in normal tissue compared to PanINs (Table 4).

3.5. CBX7 expression and tumour grading

Nuclear CBX7 expression showed an inverse correlation with higher grading ($P = 0.024$). Better differentiated tumours (Grades 1 and 2) expressed more frequently CBX7 than their poorly differentiated (Grade 3) counterparts (Table 5).

3.6. CBX7 expression and TNM classification of the tumours

The CBX7 protein expression did not show any significant association with the pT stage of the tumours ($P = 0.081$; Table 3). Moreover, no association was noted between CBX7 protein expression and lymph node status of the patients ($P = 0.12$; Table 5).

3.7. Prognostic significance

Regarding prognosis, loss of CBX7 protein expression showed a trend towards worse survival time of the patients, since five from seven (71.4%) of the patients that survived three or more years were found to have CBX7 positive tumours, while only two (28.5%) of them had CBX7 negative tumours ($P = 0.058$). Negative E cadherin expression (i.e. <10%) showed a trend toward worse survival (Fig. 3).

Table 4 – Differences in membranous E-cadherin expression between normal pancreas, PanIN and cancer.

	Normal (n 33)	PanIN (n 25)	Cancer (n 177)	P value
Mean $\pm$ SD	33.9 $\pm$ 16.2	30.0 $\pm$ 26.3	15.6 $\pm$ 16.7	<0.001
Median (min max)	30.0 (10 80)	20.0 (0 80)	10.0 (0 70)	

Table 5 – Association of CBX7 and clinico-pathological features in pancreas cancer patients.

Clinico pathological feature		Cut off (%)	Negative N (%)	Positive N (%)	P value
T stage	pT1, pT2	30	12 (12.4)	15 (22.7)	0.081
	pT3, pT4		85 (87.6)	51 (77.3)	
N stage	pN0	15	6 (20.7)	44 (35.8)	0.12
	pN1, pN2		23 (79.3)	79 (64.2)	
Tumour grade	G1, G2	15	18 (56.3)	102 (76.1)	0.024
	G3		14 (43.8)	32 (23.9)	
Survival	3 year survival rate	40	28.5%	71.4%	0.318

Fig. 1 – Examples of nuclear immunohistochemical detection of CBX7. Increased CBX7 expression in normal pancreatic tissue in (A) and reduced expression of CBX7 in pancreatic carcinoma (lower left) compared with the strong nuclear expression in small non-neoplastic ducts (upper right) in (B). Original magnification $\times 400$.

4. Discussion

In the present study, we investigated the immunohistochemical expression of the PcG CBX7 protein in 210 cases of ductal adenocarcinoma of the pancreas combined on a TMA including additional 40 PanIN 3 cases and 40 normal controls. ROC curve analysis, a method already established in clinical oncology,¹⁵ was used for the selection of clinically important cut off scores for CBX7. This was based on previous results¹⁶ showing that ROC analysis can be used as an alternative method in the selection and validation of cut off scores for immunohistochemical tumour positivity, and that the evaluation of immunoreactivity using percentage of positive tumour cells is a reproducible scoring method with a strong inter observer agreement.

In this regard, a major finding was the differential mean protein expression of CBX7 between normal pancreatic tissue and invasive adenocarcinoma and/or PanIN cases (Table 2). Mean CBX7 protein expression appeared to decrease in a stepwise manner through the progression from normal tissue to pancreatic cancer. Moreover, CBX7 expression showed an inverse correlation to the grade of the tumours, being highest in the highly differentiated carcinomas and decreasing with the dedifferentiation of the neoplasms. Therefore, our data suggest that loss of CBX7 expression correlates to a more aggressive phenotype in pancreatic adenocarcinoma. Interestingly, loss of CBX7 expression also showed a trend towards worse prognosis, since most of the long survivors in our study (survival of three or more years) had CBX7 positive tumours. These results confirm the association between lack of CBX7 and a more malignant phenotype recently reported in thyroid carcinomas by Pallante et al.,⁶ who showed that CBX7 expression progressively decreased with increasing malignancy grade and neoplasia stage in thyroid cancer patients. This finding was supported by a model of rat thyroid cells and in transgenic mice carrying thyroglobulin promoter driven oncogenes. The results reported here are also consistent with our preliminary finding of a correlation between low CBX7 expression and reduced survival in colon carcinoma (data not shown). Moreover, the association between lack of CBX7 expression and a more aggressive histotype seems to apply also to

breast, ovary and lung carcinomas (Drs. P. Pallante and G. Troncone, NOGEC, Naples, Italy).

Interestingly, also the loss of Bmi 1 expression, another member of the PcG proteins interacting with Ink4a locus, seems to be a negative prognostic factor. In fact, low Bmi 1 expression was found to correlate with adverse clinicopathological parameters in endometrial¹⁸ and renal clear cell carcinoma,¹⁹ as well as in malignant melanoma.²⁰

Since INK4a mutations are common in pancreatic cancer,²¹ it could be assumed that CBX7 is having an INK4a independent effect on pancreatic cell proliferation, as hypothesised for another PcG protein, BMI 1 on other cell systems.^{22,23} It is likely that the positive regulation of E cadherin expression by CBX7, recently demonstrated by our group,⁷ accounts for the correlation of the loss of CBX7 expression and a more aggressive phenotype of human pancreatic carcinomas. Indeed, we have shown that CBX7 is able to positively regulate E cadherin expression that plays a critical role in maintaining normal epithelial cell morphology, by interacting with Histone deacetylase 2 and inhibiting its activity on the E cadherin promoter then accounting for the correlation between the loss of CBX7 expression and a highly malignant phenotype.⁷ Moreover, in support of the above mentioned hypothesis, an additional analysis of E cadherin expression in the present series of pancreatic carcinomas showed that loss of E cadherin expression was associated with loss of CBX7 and showed a trend towards worse survival of the patients.

All these results, together with the previously reported data, showing that restoration of CBX7 expression in thyroid cancer cells reduced growth rate with a block in the G1 phase of the cell cycle, propose the CBX7 gene as a candidate tumour suppressor gene. This role appears also validated by recent results obtained in our laboratories showing a higher proliferation rate of the mouse embryonic fibroblasts (MEFs) null for CBX7 with respect to the wild type MEFs.⁶ However, other recently published data show an oncogenic activity by CBX7.^{24,25} Indeed, CBX7 cooperates with c Myc to produce highly aggressive B cell lymphomas and can initiate T cell lymphomagenesis.²⁵ Moreover, CBX7 extends the lifespan of a wide range of normal human cells and immortalises mouse fibroblasts by down regulating expression of the Ink4a/Arf locus.²⁴ Although these results seem to be contradictory at first glance, it can be

Fig. 2 – A PanIN case with moderate CBX7 expression (A). An example of a well-differentiated ductal adenocarcinoma with strong nuclear CBX7 expression (B). Examples of lower differentiated pancreatic ductal carcinomas with reduced CBX7 expression (C and D). Original magnification $\times 400$.

Fig. 3 – Kaplan-Meier survival curves demonstrating differences in survival time between patients with tumours positive and negative for CBX7 and E-cadherin.

hypothesised that the function of a certain protein can be influenced by the cellular environment, which may vary from cell to cell. There is evidence that at least some genes, like HMGA1 gene, producing proteins with oncogenic activity, can also have a tumour suppressor role as well.^{26–29} Moreover, it is known that some E2F family members can act as both oncogenes and tumour suppressor genes, depending on the cellular context.³⁰ It could thus be hypothesised that also CBX7 can exhibit both these functions, depending on the nature of other cellular events and the presence of interacting proteins. This hypothesis appears supported by our data showing that MEFs null for CBX7, in contrast to what expected from the previously pub-

lished data obtained using other cell systems,²⁴ are more susceptible to senescence (data not shown).

In conclusion, our study reveals significant differences in the mean protein expression of the PcG protein CBX7 between normal pancreatic tissue and invasive ductal adenocarcinoma of the pancreas and/or its precursor lesions (PanINs), which decreased in a stepwise manner through the progression from normal tissue to pancreatic cancer. Moreover, loss of CBX7 expression is associated with increasing malignancy grade in pancreatic adenocarcinoma suggesting that CBX7 may play an important role in pancreatic carcinogenesis.

Conflict of interest statement

None declared.

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