Novel immunohistochemical marker, integrin $\alpha_V\beta_3$, for BOP-induced early lesions in hamster pancreatic ductal carcinogenesis

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Abstract. *N*-nitrosobis(2-oxopropyl)amine (BOP)-induced pancreatic ductal carcinomas and early ductal lesions in Syrian hamsters have been reported to show histopathological resemblance to those in humans. Specific protein expression profiles have been found in human carcinomas, but a detailed molecular approach regarding the dissection of BOP-induced pancreatic carcinogenesis has yet to be determined. The present immunohistochemical study of early and advanced hamster lesions focused on five proteins reported to be overexpressed in human patients, to clarify interspecies phenotype similarity. Integrin $\alpha_V\beta_3$ was found to be overexpressed in the epithelial cells of 13 of 14 atypical hyperplasias and 6 of 6 adenocarcinomas. This overexpression was more frequent than in the remaining four proteins. However, immunoreactivity for α-enolase in epithelial cells and for kallikrein 7 and galectin-1/3 in both epithelial and stromal cells was also evident at various frequencies. Thus, similarities of tumor-associated protein expression between human and hamster pancreatic ductal lesions were confirmed, and integrin $\alpha_V\beta_3$ was identified as a potentially useful immunohistochemical marker for early lesions in hamsters.

Introduction

Pancreatic cancer is among the 10 most frequently occurring type of cancer. In Japan, pancreatic cancer is ranked fifth as a cause of cancer-related mortality. The mortality rate associated with this type of cancer also ranks high in other developed countries (1,2). The detection of pancreatic cancer at early operable stages is difficult, combined with the lack of curative treatment approaches other than complete surgical removal; thus, 5-year relative survival rates are less than 6% (3,4). Therefore, it is crucial to develop new diagnostic and preventive methods to complement any improvements in therapeutic methods for the reduction of mortality and morbidity, and to explore specific proteins overexpressed in early lesions during pancreatic carcinogenesis.

Specific protein expression profiles revealed by immunohistochemical and proteomic analyses are currently employed in the application of individualized therapy of advanced human pancreatic carcinomas (5-8). Consequently, a number of candidates of prognostic and/or predictive markers have been established (9,10). Examples expressed in early lesions show potential for the development of novel diagnostic and preventive strategies.

Chemically induced and transgenic animal models for pancreatic ductal carcinogenesis have been the target of investigation of the impact of environmental factors and the role of specific gene alterations in multistage carcinogenesis (11-15). Among the models established thus far, the *N*-nitrosobis(2-oxopropyl)amine (BOP)-induced hamster model is the first and most widely utilized based on similarities to human diseases in the morphological characteristics of, not only advanced cancers, but also early ductal lesions, as well as pivotal genetic alterations, including *K-ras* and *p16* (13,16-18). In particular, it is anticipated that molecular profiles are equivalent in the early stages. Although changes in the protein expression have been reported (5-8) in pancreatic carcinomas in humans, molecular details of BOP-induced pancreatic early lesions in hamsters have yet to be investigated. In the present study, an immunohistochemical analysis was conducted on pancreatic carcinomas and early lesions induced in BOP-treated hamsters, focusing on proteins already reported to be altered in human cases. As a result, integrin $\alpha_V\beta_3$ was found to be more frequently expressed in both pancreatic carcinomas and its precursors than the four remaining proteins investigated. The results obtained showed multiple similarities in tumor-associated protein expression between human and hamster pancreatic ductal lesions.

Materials and methods

Animals. A total of 12 female Syrian golden hamsters at 5 weeks of age were purchased from Japan SLC (Shizuoka, Japan). The animals were housed 3 to a plastic cage with soft woodchip bedding (Japan SLC) in an air-conditioned animal room maintained at 22±2˚C and 60±5% relative humidity, with

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Abbreviations: BOP, *N*-nitrosobis(2-oxopropyl)amine

Key words: integrin $\alpha_V\beta_3$, Syrian hamster, pancreatic ductal carcinoma, early stage, *N*-nitrosobis(2-oxopropyl)amine

Table I. Antibodies and antigen retrieval methods for immunochemistry.

Protein	Antibodies	Antigen retrieval
Integrin $\alpha_V\beta_3$	Anti-human integrin $\alpha_V\beta_3$ monoclonal (Clone LM609; Chemicon International, Temecula, CA, USA)	Autoclaved for 10 min at 121˚C in 10 mM Tris-HCl buffer, including 1 mM EDTA (pH 9.0)
Kallikrein 7	Anti-kallikrein 7 goat polyclonal (R&D Systems, Minneapolis, MN, USA)	Autoclaved for 10 min at 121˚C in 10 mM citrate buffer (pH 6.0)
Galectin 1	Anti-galectin-1 rabbit polyclonal (Protein Tech Group, Chicago, IL, USA)	Autoclaved for 15 min at 121˚C in 10 mM citrate buffer (pH 6.0)
Galectin 3	Anti-galectin-3 rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA, USA)	Autoclaved for 10 min at 121˚C in 10 mM citrate buffer (pH 6.0)
α-Enolase	Anti-α-enolase rabbit polyclonal (Aviva Systems Biology LLC., San Diego, CA, USA)	Autoclaved for 10 min at 121˚C in distilled water

a 12:12 h light:dark cycle. A basal diet (CE-2; CLEA Japan, Tokyo, Japan) and water were available *ad libitum* throughout the experiment.

Treatment for pancreatic tumor induction. Following an acclimatization period of 1 week, 9 hamsters were subcutaneously injected with BOP (Nacalai Tesque, Kyoto, Japan) in saline at 10 mg/kg body weight every other day for a total of four times. Additionally, 3 hamsters treated with saline were maintained as control animals. The experimental protocols were approved by the Committee for Ethics of Animal Experimentation of the National Cancer Center and were carried out according to the Guidelines for Animal Experiments.

Histopathological examination. At 23 weeks of age, all 12 hamsters were sacrificed and each pancreas was removed, fixed in 10% buffered formalin, processed for embedding in paraffin, sectioned and stained with hematoxylin and eosin for histopathological evaluation. Pancreatic ductal proliferative lesions were classified as atypical hyperplasias (AHs) and adenocarcinomas (ACs) according to the criteria previously described (19). Briefly, AH consisted of ductules with increased cell proliferation but minimal nuclear atypia and no loss of polarity. The typical AC had a distinct tubular, cribriform or anaplastic pattern with severely atypical columnar or cuboidal epithelia.

Immunohistochemical staining. A total of five target proteins, known to be specifically expressed in pancreatic carcinoma tissues or cell lines established from pancreatic carcinomas, were selected, as previously reported (5-8). Antibodies and antigen retrieval methods used in this study are listed in Table I. The role played by each was: integrin $\alpha_V\beta_3$, a transmembrane glycoprotein, is involved in cell-to-cell and cell-to-matrix interactions and thus may contribute to cancer progression, invasiveness and metastasis (5); kallikrein 7, a member of the serine protease family, enhances pancreatic cancer cell invasion by shedding E-cadherin (6); galectin-1, a soluble β-galactoside-binding animal lectin, modulates cell adherence and plays a role in tumor progression (7); galectin-3, another soluble β-galactoside-binding animal lectin, modulates cell adherence (8); and α-enolase, a glycolytic enzyme, is involved in the conversion of 2-phosphoglycerate phosphoenolpyruvate (7,20). The streptavidin-biotin-peroxidase complex method (StreptABComplex/HRP; DakoCytomation, Glostrup, Denmark) was employed to determine the expression and localization of each protein, and the sections were lightly counterstained with hematoxylin for microscopic examination. Negative controls without primary antibody reactions were set for each protein using serial sections. The staining intensity of each protein was analyzed with reference to the positivity rate in all epithelial and stromal cells in AHs and ACs and represented as <10%, negative (-); 10-70%, moderately positive (+); and >70%, strongly positive (++).

Results

Pancreatic ductal lesions induced by BOP. A total of 14 AHs and 6 ACs were induced in the 9 hamsters treated with BOP, whereas no lesions were found in the 3 animals without carcinogen exposure. The incidences and multiplicities (mean ± SD) of AHs and ACs were 89%, 1.6±1.1 and 44%, 0.7±0.9, respectively. Only 1 case of AC showed poorly differentiated characteristics, while the remaining cases were moderately differentiated tubular ACs. All cases harbored abundant stroma (data not shown).

Immunohistochemical findings for the five proteins analyzed

Normal pancreas (Fig. 1). Pancreatic ductal and ductular cells as well as islet cells showed weak immunohistochemical staining for α-enolase in hamsters without BOP treatment. The reactions in ductal and islet cells were cytoplasmic and essentially homogeneous. Integrin $\alpha_V\beta_3$, kallikrein 7 and galectin 1/3 were almost negative in the normal pancreatic tissues.

Atypical hyperplasias (Fig. 2). Integrin $\alpha_V\beta_3$ and α-enolase were expressed predominantly in the epithelial components of AHs, whereas kallikrein 7 and galectin 1/3 were expressed in both the epithelial and adjacent stromal elements. The morphological characteristics of the numerous stromal cells positive for kallikrein 7 and/or galectin 1/3 characterized these cells as fibroblasts. Regarding subcellular staining, integrin $\alpha_V\beta_3$ was mostly localized in the cell cytoplasm, while appearing to aggregate with a granular pattern towards the apex from the nuclei in the epithelial cells. Concerning

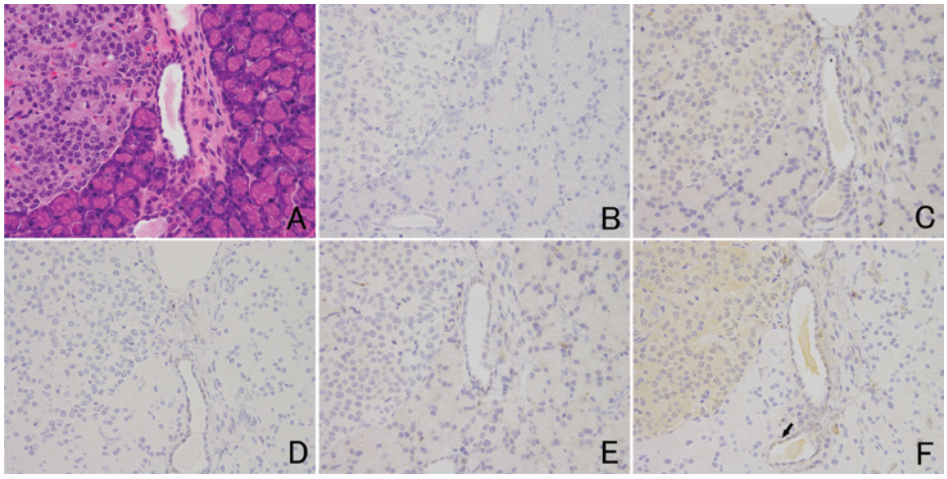

Figure 1. Normal hamster pancreatic tissue, including ductules (center panels), an islet (left panels) and acini (right panels). (A) H&E and immunohistochemistry for (B) integrin $\alpha_V\beta_3$, (C) kallikrein 7, (D) galectin 1, (E) galectin 3 and (F) α-enolase. The proteins were essentially negative, except for weak cytoplasmic positivity for (F) α-enolase in the islet and ductular cells (arrow). Original magnification, x200.

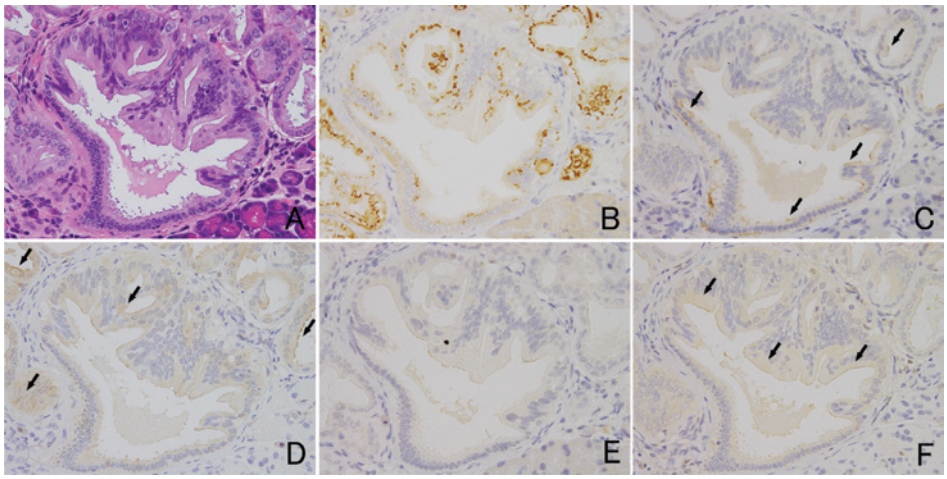

Figure 2. Atypical hyperplasia in a BOP-treated hamster. (A) H&E and immunohistochemistry for (B) integrin $\alpha_V\beta_3$, (C) kallikrein 7, (D) galectin 1, (E) galectin 3 and (F) α-enolase. (B) Integrin $\alpha_V\beta_3$ localization, particularly in the cytoplasm, with a granular pattern in the epithelial cells. In this case, (E) galectin 3 appears negative. (C, D and F) Other proteins are almost uniformly positive in the epithelial cytoplasm and/or are localized on the apical surface (arrows). Original magnification, x200.

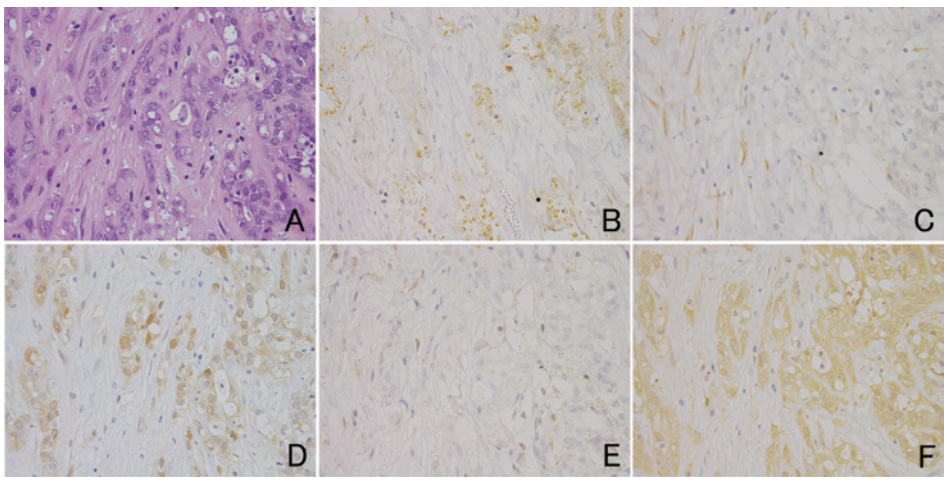

Figure 3. Adenocarcinoma in a BOP-treated hamster. (A) H&E and immunohistochemistry for (B) integrin $\alpha_V\beta_3$, (C) kallikrein 7, (D) galectin 1, (E) galectin 3 and (F) α-enolase. (B, D and F) Integrin $\alpha_V\beta_3$, galectin 1 and α-enolase are mainly positive in the epithelial tumor cells, and (C and E) kallikrein 7 and galectin 3 are mainly reactive in the stromal cells. Original magnification, x200.

Table II. Incidences and intensities of immunostaining in positive AHs and ACs.

Protein	Lesion	Location	Incidence (%)	
			Intensity (+)	Intensity (++)
Integrin $\alpha_V\beta_3$	AH (n=14)	Epithelium	8 (57)	5 (36)
		Stroma	0	0
	AC (n=6)	Epithelium	4 (67)	2 (33)
		Stroma	0	0
Kallikrein 7	AH (n=14)	Epithelium	6 (43)	0
		Stroma	4 (28)	4 (28)
	AC (n=6)	Epithelium	4 (66)	0
		Stroma	0	6 (100)
Galectin 1	AH (n=14)	Epithelium	0	3 (21)
		Stroma	1 (7)	0
	AC (n=6)	Epithelium	4 (67)	0
		Stroma	1 (17)	0
Galectin 3	AH (n=14)	Epithelium	2 (14)	1 (7)
		Stroma	1 (7)	1 (7)
	AC (n=6)	Epithelium	0	0
		Stroma	2 (33)	0
α-Enolase	AH (n=14)	Epithelium	11 (79)	0
		Stroma	0	0
	AC (n=6)	Epithelium	6 (100)	0
		Stroma	0	0

AH, atypical hyperplasia; AC, adenocarcinoma; +, moderately positive; ++, strongly positive.

kallikrein 7, galectin 1/3 and α-enolase, positivity was found almost uniformly in the cell cytoplasm and/or was localized on the apical surfaces.

Adenocarcinomas (Fig. 3). Similar to the AHs, integrin $\alpha_V\beta_3$ and α-enolase proved to be positive in the epithelia, while kallikrein 7 and galectin 1 were observed in the epithelial and stromal cells. Galectin 3 was stained only in the stromal cells. Subcellular staining patterns were similar to those in AHs.

Immunostaining incidences and intensities for the five proteins. Staining incidences and intensities for each protein were evaluated based on the positivity rates for cells (Table II). A total of 13 of14 AHs (93%) and 6 of 6 ACs (100%) were positive for integrin $\alpha_V\beta_3$. Consequently, the incidence of integrin $\alpha_V\beta_3$ was higher compared to the remaining four proteins, and in 5 of the AHs (36%) and 2 of the ACs (33%), the grading was strongly positive (++). Staining was found to be negative in the stroma. By contrast, kallikrein 7 was predominantly expressed in the stroma of 8 of 14 AHs (57%) and 6 of 6 ACs (100%), and the epithelial cells of 6 of 14 AHs (43%) were also stained. In 4 AHs (28%) and 6 ACs (100%), stromal cells were graded as strongly positive (++). Galectin 1 was predominantly expressed in the epithelium of 3 of 14 AHs (21%) and 4 of 6 ACs (67%), and all the AHs were graded as strongly positive (++). Galectin 3 was also expressed in both the epithelium and stroma of AHs (3/14, 21% and 2/14, 14%, respectively), and the stroma of ACs (2/6, 33%). Among the positive cases, 1 AH case showed strongly positive in both the epithelial and stroma cells. Expression of α-enolase was observed predominantly in the epithelial cells of 11 of 14 AHs (79%) and 6 of 6 ACs (100%). Staining intensity was moderately positive (+).

Discussion

The present immunohistochemical analysis focusing on five proteins reported to be overexpressed in human pancreatic carcinomas showed that integrin $\alpha_V\beta_3$ was found to be frequently and strongly expressed in BOP-induced hamster pancreatic early ductal lesions and carcinomas. This expression is in agreement with its reported promotion of cell migration and proliferation (21,22). Subcellular localization aggregated in the cytoplasm of epithelial cells has also been reported in human pancreatic cancer cases (5). Although a slight expression of integrin $\alpha_V\beta_3$ was found in pancreatic ductal hyperplasias, which lack cellular atypia and are thought to be initial histological focal changes, the frequency was lower than that in more advanced lesions (data not shown). The significance of the overexpression of integrin $\alpha_V\beta_3$ in pancreatic ductal carcinogenesis has yet to be elucidated. However, dysregulation in protein transportation and/or degradation functions may occur in the early stages of pancreatic ductal carcinogenesis in hamsters and humans. The relationship between integrin $\alpha_V\beta_3$-positivity and parameters, such as proliferation, apoptosis and invasiveness, has yet to be investigated. However, in human cases, 58% of pancreatic carcinomas showed positive staining and the frequency was significantly higher in primary tumors with lymph node metastasis (5). Recently, integrin $\alpha_V\beta_3$ was also studied as a target molecule for imaging diagnosis

in mammary carcinoma (23), and this BOP-induced hamster model may aid in the pre-clinical screening of integrin $\alpha_V\beta_3$ and other molecular-targeted probes and/or medicines.

Kallikrein 7 is a chymotrypsin-like serine protease, originally purified from human skin, which is specifically expressed in keratinizing squamous epithelia (24), and is involved in cell invasion by cleavage of the extracellular domain of adhesion molecules, such as E-cadherin. In the present study, Kallikrein 7 was found to be predominantly expressed in stromal cells of both AHs and ACs. Moderate-to-intense staining for kallikrein 7 has been reported in the majority of human pancreatic carcinomas, but this staining is distributed among the majority of the tumor cells (6). Although the cause of such variation remains to be determined, cytoplasmic positivity and/or localization on the apical surfaces was evident in the epithelial cells of some of our AHs and ACs in hamsters.

Galectin 1/3 are members of the family of β-galactoside-binding animal lectins (25) and play a role in a variety of cell functions, including proliferation, migration and adhesion characteristics (26,27). In this study, the expression of galectin 1/3 was observed at lower frequencies than the remaining three proteins in the epithelial and stromal cells in AHs, whereas it was strongly expressed in epithelial and stromal cells, respectively, in ACs. In human carcinoma cases, however, the opposite phenomenon has been described, with galectin 1 being stronger in the stroma and galectin 3 in the epithelial elements (7,8). The causes for this phenomenon remain to be determined.

α-Enolase, a glycolytic enzyme, is involved in the conversion of 2-phosphoglycerate to phosphoenolpyruvate in the glycolytic pathway (28). In the present analysis, α-enolase showed the second most frequent expression in the epithelium of both AHs and ACs. On the other hand, normal hamster pancreatic islets and acinar and ductal epithelium cells were weakly positive, partially consistent with a previous report (29).

In conclusion, some similarities to the human tumor-associated protein expression were confirmed in hamster pancreatic ductal lesions. The addition of molecules may also be identified by a global analysis using cDNA microarrays and/or proteomic approaches. Additional studies using hamsters may allow for the discovery of target molecules for practical diagnostic and preventive methods for human early pancreatic carcinomas.

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