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Clinical Significance of Ephrin (Eph)-A1, -A2, -A4, -A5 and -A7 Receptors in Pancreatic Ductal Adenocarcinoma

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Abstract Ephrin (Eph) receptors have been reported to be frequently overexpressed in a wide variety of cancer types, being associated with tumor growth, invasion, metastasis and angiogenesis. The aim of the present study was to evaluate the clinical significance of Eph-A1, -A2, -A4, -A5 and -A7 expression in pancreatic ductal adenocarcinoma. Eph-A1, -A2, -A4, -A5 and -A7 expression and staining intensity were assessed immunohistochemically in tumoral samples of 67 pancreatic adenocarcinoma patients and were statistically analyzed in relation to clinicopathological characteristics, tumor proliferative capacity and patients' survival. Eph receptors were abundantly expressed in pancreatic ductal adenocarcinoma cases examined. Eph-A1 staining intensity was significantly associated with tumor size (pT, p=0.008) and tumor histopathological stage (pStage, p=0.012). Eph-A2 expression was significantly associated with patients' age (p=0.007), while Eph-A4 and Eph-A5 with tumor proliferative capacity (p=0.019 and p=0.011, respectively). Pancreatic adenocarcinoma patients with moderate/intense Eph-A5 or Eph-A7 staining presented significantly shorter survival times compared to those with negative/mild one (log-rank test, p=0.024 and p=0.009, respectively). Multivariate analysis identified Eph-A5 and Eph-A7 staining intensity as independent prognostic factors (p=0.048 and p=0.004, respectively). In conclusion, the present study revealed that Eph receptors were associated with pancreatic cancer characteristics, supporting evidence for their potential clinical application in management and prognosis of pancreatic adenocarcinoma patients.

Keywords Ephrins · Clinical significance · Clinicopathological parameters · Immunohistochemistry · Pancreatic adenocarcinoma · Prognostic marker

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Introduction

Ephrin (Eph) receptors constitute the largest sub-family of receptor tyrosine kinases, being divided into two subgroups, EphA and EphB, based on their ligand-binding-affinity and structure of the extracellular domain [1, 2]. Nine EphA (EphA1-9) and six EphB (EphB1–6) receptors have been identified to date. Their membrane-anchored ligands, the ephrins (ephs), are also divided into two subgroups, ephA and ephB, which preferentially bind to EphA and EphB receptors, respectively [1–4]. Eph receptors and eph ligands have been shown to form a vital cell communication system capable of bi-directional signaling [1, 2]. Ephs/ephs signaling has initially been shown to participate in a wide spectrum of developmental processes, being capable of regulating cellular adhesion,

migration or chemo-repulsion and tissue/cell boundary formation [1–5]. Recent evidence has further extended the role of Eph receptors and their ligands as critical regulators of vascular remodelling during embryogenesis and tumor neovascularization. Thus, it is not surprising that beyond their initial role in developmental processes, Eph receptors and their ligands have been involved in a broad range of processes directly related with tumorigenesis and metastasis, including cell attachment and shape, migration and angiogenesis [5–10]. Moreover, it should be noted that unlike traditional oncogenes that often function only in tumor cells, Eph receptors mediate cell to cell interactions both in tumor cells and in tumor microenvironment, namely tumor stroma and vasculature. Thus, Eph receptors have been considered as attractive targets for drug design, as targeting these molecules could simultaneously inhibit several aspects of tumor progression [5–10].

Pancreatic cancer is one of the most lethal malignant tumors presenting extremely poor prognosis, as survival longer than 5 years is actually rare. Indeed, tumor resection is performed in 9–36% of patients and the 5-year survival rate of patients who have undergone resection is only 19–24% [11, 12]. Moreover, the clinical presentation of chronic pancreatitis is often confused with that of pancreatic cancer, thus differential diagnosis remains complicated despite the significant progress supported by imaging techniques [12, 13]. Hence, there is a strong demand for novel specific markers in respect to prognosis and management of pancreatic adenocarcinoma patients. It has also been shown that combined surgery and chemotherapy based on 5-fluorouracil or gemcitabine, with or without radiation treatment, is not potentially capable of contributing to significant survival benefit [13]. Thus, the establishment of alternative

Table 1 Associations of Eph-A1 expression and staining intensity with clinicopathological characteristics in pancreatic adenocarcinoma patients

Clinicopathological parameters	Eph-A1 expression			Eph-A1 intensity		
	Low (%)	High (%)	*p*-value	Negative/mild (%)	Moderate/intense (%)	p-value
Patients (*N*=67)	37(55)	30(45)		28(42)	39(58)	
Age			0.682			0.191
< 66	13(19)	12(18)		13(19)	12(18)	
≥ 66	24(36)	18(27)		15(23)	27(40)	
Gender			0.371			0.987
Men	22(33)	21(31)		18(27)	25(37)	
Women	15(22)	9(14)		10(15)	14(21)	
Histopathological Grade			0.234			0.549
Well	4(6)	8(12)		5(7)	7(11)	
Moderate	26(39)	18(27)		20(30)	24(36)	
Poor	7(10)	4(6)		3(4)	8(12)	
pT classification			0.376			0.008
T1-2	5(7)	6(9)		2(3)	9(13)	
T3	28(42)	18(27)		25(37)	21(32)	
T4	4(6)	6(9)		1(2)	9(13)	
pN classification			0.514			0.072
N0	18(27)	17(25)		11(17)	24(36)	
N1	19(28)	13(20)		17(25)	15(22)	
pM classification			0.412			0.731
M0	34(51)	29(43)		26(39)	37(55)	
M1	3(4)	1(2)		2(3)	2(3)	
pStage			0.276			0.012
I	3(4)	6(9)		1(2)	8(12)	
II	27(40)	17(25)		24(36)	20(30)	
III-IV	7(11)	7(11)		3(4)	11(16)	
Ki-67 protein statement			0.938			0.528
Below mean (≤25%)	25(37)	20(30)		20(30)	25(37)	
Over mean (>25%)	12(18)	10(15)		8(12)	14(21)	

therapeutic approaches for the treatment of pancreatic cancer remains a great challenge.

Accumulative evidence has demonstrated that Eph receptors are overexpressed in a variety of tumors, being associated with important clinicopathological parameters and patients' survival [5–10]. However, most of the available data so far is mainly restricted to Eph-A1 and -A2 receptors and does not concern other members of EphA family. Moreover, the clinical significance of Eph receptors in pancreatic adenocarcinoma patients remains to be clarified. In view of above considerations, the present study aimed to assess the immunohistochemical expression of Eph-A1, -A2, -A4, -A5 and -A7 in tumoral specimens obtained from pancreatic adenocarcinoma patients. We also aimed to evaluate the association of Eph-A1, -A2, -A4, -A5 and -A7 expression with clinicopathological characteristics, tumor proliferative capacity and patients' survival.

Materials and Methods

Clinical Material

Sixty-seven pancreatic adenocarcinoma specimens obtained from equal number of patients who underwent surgical resection due to pancreatic cancer were included in the present study. The study was approved by the ethical committee of Laikon General Hospital. None of the patients received any kind of anti-cancer treatment prior to surgery. Forty-three of the patients were men (64%) and 24 were women (36%) with a mean age of 66.3±9.4 years (range 43–84 years). Three levels of differentiation were used to clarify histological grading according to the criteria described by WHO [14] as: well in 12 (18%), moderately in 44 (66%) and poorly differentiated in 11 (16%) cases. Tumors staging was assessed using the 5th edition of the

Table 2 Associations of Eph-A2 expression and staining intensity with clinicopathological characteristics in pancreatic adenocarcinoma patients

Clinicopathological parameters	Eph-A2 expression			Eph-A2 intensity		
	Low (%)	High (%)	p-value	Negative/mild (%)	Moderate/intense (%)	p-value
Patients (*N*=67)	33(49)	34(51)		44(66)	23(34)	
Age			0.007			0.450
< 66	7(10)	18(27)		15(22)	10(15)	
≥ 66	26(39)	16(24)		29(43)	13(19)	
Gender			0.927			0.506
Men	21(31)	22(33)		27(40)	16(24)	
Women	12(18)	12(18)		17(26)	7(10)	
Histopathological Grade			0.328			0.512
Well	8(12)	4(6)		7(10)	5(7)	
Moderate	19(28)	25(37)		31(46)	13(19)	
Poor	6(9)	5(8)		6(9)	5(7)	
pT classification			0.921			0.294
T1-2	6(9)	5(7)		6(9)	5(7)	
T3	22(33)	24(36)		33(49)	13(10)	
T4	5(7)	5(7)		5(7)	5(7)	
pN classification			0.544			0.306
N0	16(24)	19(28)		21(34)	14(21)	
N1	17(25)	15(23)		23(34)	9(13)	
pM classification			0.975			0.685
M0	31(46)	32(48)		41(61)	22(33)	
M1	2(3)	2(3)		3(4)	1(2)	
pStage			0.953			0.199
I	4(6)	5(7)		4(6)	5(7)	
II	22(33)	22(33)		32(48)	12(18)	
III-IV	7(11)	7(11)		8(12)	6(9)	
Ki-67 protein statement			0.663			0.762
Below mean (≤25%)	23(34)	22(33)		29(43)	16(24)	
Over mean (>25%)	10(15)	12(18)		15(22)	7(11)	

Tumor, Node, Metastasis (TNM) system according to the Union Internationale Contra la Cancrum (UICC) and the American Joint Committee on Cancer (AJCC) [15]. In fact, tumors were classified as: T1 in 3 (4%), T2 in 8 (12%), T3 in 46 (69%) and T4 in 10 (15%) cases. Thirty-five patients (52%) were node negative (N0), and 32 (48%) were regional node positive (N1). Organ metastasis was noted in 4 (6%) out of 67 patients examined. The patients were followed up until death for a time interval of 4 up to 21 months (median value 8 months).

Immunohistochemistry

Immunostainings for Eph-A1, -A2, -A4, -A5 and -A7 were performed on formalin-fixed, paraffin-embedded tissue sections using commercially available rabbit polyclonal Eph-A1 (S-20), Eph-A2 (H-120), Eph-A4 (H-77), Eph-A5 (C-16) and Eph-A7 (C-19) primary IgG antibodies (Santa Cruz Biochemicals, Santa Cruz, CA, USA). Briefly, 4 μm thick tissue sections were dewaxed in xylene and were brought to water through graded alcohols. Antigen retrieval (citrate buffer at pH 6.1 and microwave heating) was then performed. To remove the endogenous peroxidase activity, sections were then treated with freshly prepared 0.3% hydrogen peroxide in methanol in the dark, for 30 minutes (min), at room temperature. Non-specific antibody binding was then blocked using Snipper, a specific blocking reagent for rabbit primary antibodies (Sniper, Biocare Medical, Walnut, Creek, CA, USA) for 5 min. The sections were then incubated for 1 hour (h), at room temperature, with primary antibodies, diluted 1:100 in phosphate buffered saline (PBS). After washing three times with PBS, sections were incubated at room temperature with biotinylated linking reagent (Biocare Medical) for 10 min, followed by

Table 3 Associations of Eph-A4 expression and staining intensity with clinicopathological characteristics in pancreatic adenocarcinoma patients

Clinicopathological parameters	Eph-A4 expression			Eph-A4 intensity		
	Low (%)	High (%)	p-value	Negative/mild (%)	Moderate/intense (%)	p-value
Patients (N=67)	38(57)	29(43)		24(36)	43(64)	
Age			0.150			0.281
< 66	17(25)	8(12)		11(17)	14(21)	
≥ 66	21(31)	21(31)		13(19)	29(43)	
Gender			0.841			0.455
Men	24(36)	19(28)		14(21)	29(43)	
Women	14(21)	10(15)		10(15)	14(21)	
Histopathological Grade			0.264			0.507
Well	9(13)	3(5)		6(9)	6(9)	
Moderate	22(33)	22(33)		14(21)	30(45)	
Poor	7(10)	4(6)		4(6)	7(10)	
pT classification			0.318			0.311
T1-2	8(12)	3(4)		6(9)	5(7)	
T3	26(39)	20(30)		14(21)	32(48)	
T4	4(6)	6(9)		4(6)	6(9)	
pN classification			0.159			0.432
N0	17(25)	18(27)		11(17)	24(36)	
N1	21(31)	11(16)		13(19)	19(28)	
pM classification			0.446			0.541
M0	35(52)	28(42)		22(33)	41(61)	
M1	3(4)	1(2)		2(3)	2(3)	
pStage			0.733			0.637
I	6(9)	3(4)		4(6)	5(7)	
II	25(37)	19(28)		14(21)	30(45)	
III-IV	7(11)	7(11)		6(9)	8(12)	
Ki-67 protein statement			0.019			0.632
Below mean (≤25%)	30(45)	15(22)		17(26)	28(42)	
Over mean (>25%)	8(12)	14(21)		7(10)	15(22)	

incubation with peroxidase-conjugated streptavidin label (Biocare Medical) for 10 min. The resultant immune peroxidase activity was developed in 0.5% 3,3'-diaminobenzidine hydrochloride (DAB; Sigma, Saint Louis, MO, USA) in PBS containing 0.03% hydrogen peroxide for 3 min. Sections were counterstained with Harris' hematoxylin and mounted in Entellan (Merck, Darmstadt, Germany). Appropriate negative controls were performed by omitting the primary antibody and/or substituting it with an irrelevant anti-serum. As positive control, pancreatic cancer tissue sections with known increased EphA positivity [16], as also human brain tissues (data not shown) were used. The tumor cells proliferative capacity was assessed immunohistochemically, using a mouse *anti*-human Ki-67 antigen; IgG_{1k} antibody (clone MIB-1, Dakopatts, Glostrup, Denmark) as previously described [17, 18].

The percentages of positively stained cells were obtained by counting at least 1000 cells in each case by two independent observers (S.T. and A.Z.-S.) blinded to the clinical data with complete observer agreement. Specimens were considered "positive" for Eph receptors when more than 5% of tumor cells within the section were positively stained [17–19]. Specimens were characterized to present "high expression" for Eph receptors when the percentage of the positively stained cells exceeds the mean percentage value [17–19]. In Eph-positive cases, the intensity of staining was also estimated and graded in a three step scale as mild (+), moderate (++) and intense (+++) [17–19].

Statistical Analysis

Chi-square tests were used to assess the statistical significance of the association of Eph-A1, -A2, -A4, -A5 and -A7 expression and staining intensity with clinicopathological variables. Spearman's rank correlation analysis was used to examine the linear relationship between Eph-A1, -A2, -A4,

Table 4 Associations of Eph-A5 expression and staining intensity with clinicopathological characteristics in pancreatic adenocarcinoma patients

Clinicopathological parameters	Eph-A5 expression			Eph-A5 intensity		
	Low (%)	High (%)	p-value	Negative/mild (%)	Moderate/intense (%)	p-value
Patients (*N*=67)	30(45)	37(55)		21(31)	46(67)	
Age			0.358			0.317
< 66	13(19)	12(18)		6(9)	19(28)	
≥ 66	17(26)	25(37)		15(22)	27(40)	
Gender			0.896			0.403
Men	19(28)	24(36)		15(22)	28(42)	
Women	11(17)	13(19)		6(9)	18(27)	
Histopathological Grade			0.586			0.501
Well	4(6)	8(12)		4(6)	8(12)	
Moderate	20(30)	24(36)		12(18)	32(48)	
Poor	6(9)	5(7)		5(7)	6(9)	
pT classification			0.214			0.540
T1-2	6(9)	5(7)		5(7)	6(9)	
T3	22(33)	24(36)		13(19)	33(49)	
T4	2(3)	8(12)		3(4)	7(10)	
pN classification			0.188			0.110
N0	13(19)	22(33)		14(21)	21(31)	
N1	17(26)	15(22)		7(10)	25(37)	
pM classification			0.412			0.163
M0	29(43)	34(51)		21(31)	42(63)	
M1	1(2)	3(4)		0(0)	4(6)	
pStage			0.135			0.206
I	5(7)	4(6)		5(7)	4(6)	
II	22(33)	22(33)		13(19)	31(46)	
III-IV	3(4)	11(16)		3(4)	11(16)	
Ki-67 protein statement			0.011			0.953
Below mean (≤25%)	25(37)	20(30)		14(21)	31(46)	
Over mean (>25%)	5(7)	17(25)		7(10)	15(22)	

-A5 and -A7 percentage expression. Survival curves were constructed using the Kaplan-Meier method and compared by the log rank test. The influence of Eph-A1, -A2, -A4, -A5 and -A7 expression and staining intensity as prognostic factors on patients' survival was evaluated using Cox regression analysis. All reported *p*-values are based on two-sided hypotheses. A *p*-value less than 0.05 was considered the limit of statistical significance. SPSS for Windows Software was used for all analyses (SPSS Inc., 2003, Chicago, USA).

Results

Eph receptors were abundantly and haphazardly expressed in pancreatic ductal adenocarcinoma cases examined, presenting mainly cytoplasmic and occasionally membraneous pattern of staining. Approximately a half of pancreatic ductal adenocarcinoma cases presented high expression or moderate to intense staining intensity for Eph receptors (Tables 1, 2, 3, 4 and 5). Representative immunostainings for Eph-A1, -A2, -A4, -A5 and -A7 are illustrated in Fig. 1a-e. Non-cancerous sites of pancreatic tissues were mostly negative or showed a very weak cytoplasmic staining (data not shown).

In cross-tables, Eph-A1, -A2, -A4, -A5 and -A7 expression was not significantly associated with the clinicopathological characteristics examined (Tables 1, 2, 3, 4 and 5), except for an association between Eph-A2 expression and patients' age (Table 2, $p=0.007$). Eph-A1, -A2 and -A4 expression was not associated with tumors' proliferative capacity (Tables, 1, 2 and 3), whereas pancreatic adenocarcinoma cases with enhanced Eph-A5 and -A7 expression presented significantly increased tumor cells proliferative capacity (Tables 4 and 5,

Table 5 Associations of Eph-A7 expression and staining intensity with clinicopathological characteristics in pancreatic adenocarcinoma patients

Clinicopathological parameters	Eph-A7 expression			Eph-A7 intensity		
	Low (%)	High (%)	p-value	Negative/mild (%)	Moderate/intense (%)	p-value
Patients (*N*=67)	36(54)	31(46)		29(43)	37(55)	
Age			0.826			0.650
< 66	13(19)	12(18)		10(15)	15(22)	
≥ 66	23(34)	19(28)		19(28)	22(33)	
Gender			0.572			0.131
Men	22(33)	21(31)		16(24)	27(30)	
Women	14(21)	10(15)		13(10)	10(15)	
Histopathological Grade			0.931			0.755
Well	7(11)	5(7)		7(10)	5(7)	
Moderate	23(34)	21(31)		18(27)	25(37)	
Poor	6(9)	5(7)		4(6)	7(10)	
pT classification			0.967			0.092
T1-2	6(9)	5(7)		7(10)	4(6)	
T3	25(37)	21(31)		18(27)	28(42)	
T4	5(7)	5(7)		4(6)	5(7)	
pN classification			0.924			0.415
N0	19(28)	16(24)		17(25)	18(27)	
N1	17(25)	15(22)		12(18)	19(28)	
pM classification			0.378			0.707
M0	33(49)	30(45)		28(42)	34(51)	
M1	3(4)	1(2)		1(2)	3(4)	
pStage			0.632			0.193
I	6(9)	3(4)		6(9)	3(4)	
II	22(33)	22(33)		18(27)	26(39)	
III-IV	8(12)	6(9)		5(7)	8(12)	
Ki-67 protein statement			0.141			0.351
Below mean (≤25%)	27(40)	18(27)		20(30)	25(37)	
Over mean (>25%)	9(13)	13(19)		9(13)	12(18)	

p=0.019 and p=0.011, respectively). Eph-A1 staining intensity was significantly associated with tumor size (pT, p=0.008) and pStage (p=0.012), presenting also a trend of correlation with lymph node positivity (pN, p=0.072). No significant associations between Eph-A2, -A4, -A5 and -A7 staining intensity and the clinicopathological characteristics examined, as well as tumor cells proliferative capacity (Tables 1, 2, 3, 4 and 5) were noted, except for a trend of correlation between Eph-A7 and tumor size (Table 5, pT, p=0.092).

The Spearman's rank correlation coefficient was calculated to evaluate the linear relationships amongst the percentage expression of Eph-A1, -A2, -A4, -A5 and -A7 (percentage of positively stained tumor cells) (Table 6). Significant positive correlations were obtained between Eph-A2 percentage expression and that of Eph-A4, -A5 and -A7 (Table 6, r_s= 0.259 p=0.034, r_s=0.247 p=0.044, r_s=0.301 p=0.013). Eph-A4 percentage expression was strongly correlated with that of Eph-A5 and -A7 (Table 6, r_s=0.332 p=0.006, r_s= 0.428 p=0.0003). A significant positive association between Eph-A5 and Eph-A7 percentage expression was also noted (Table 6, r_s=0.321 p=0.008).

The Kaplan-Meier product-limit method for overall survival analysis according to Eph-A1, -A2, -A4, -A5 and -A7 expression (low *vs* high Eph-A1, -A2, -A4, -A5 and -A7 expression) in pancreatic adenocarcinoma specimens did not reveal significant associations (log-rank test, p=0.427, p= 0.837, p=0.517, p=0.630 and p=0.181, respectively). Univariate analysis also showed no significant differences in the survival times of pancreatic adenocarcinoma patients with respect to Eph-A1, -A2 and -A4 staining intensity (negative/mild *vs* moderate/intense) (log-rank test, p=0.884, p=0.993 and p=0.406, respectively). Pancreatic adenocarcinoma patients presenting moderate/intense Eph-A5 and Eph-A7 staining intensity were significantly characterized by shorter survival times compared to those with negative/mild staining (log-rank test, p=0.024 and p=0.009, Fig. 2a and b, respectively). Multivariate survival Cox regression analysis also showed statistical significance for Eph-A5 and Eph-A7 staining intensity (p=0.048 and p=0.004, respectively).

Discussion

In the last few years, accumulative evidence has suggested that Eph receptors and their ligands are frequently overexpressed in a variety of human malignant tumors including oesophageal, breast, small-cell lung, gynaecological and gastrointestinal carcinomas, melanomas and neuroblastomas [5–10]. Recent studies have also provided substantial evidence that Eph receptors may play a role in pancreatic cancer biology. In fact, posttranscriptional suppression of Eph-A2 expression was found to affect the malignant cellular phenotype of pancreatic adenocarcinoma cells [20]. Eph-A2 overexpression was shown to enhance pancreatic adenocarcinoma cellular invasiveness by in-

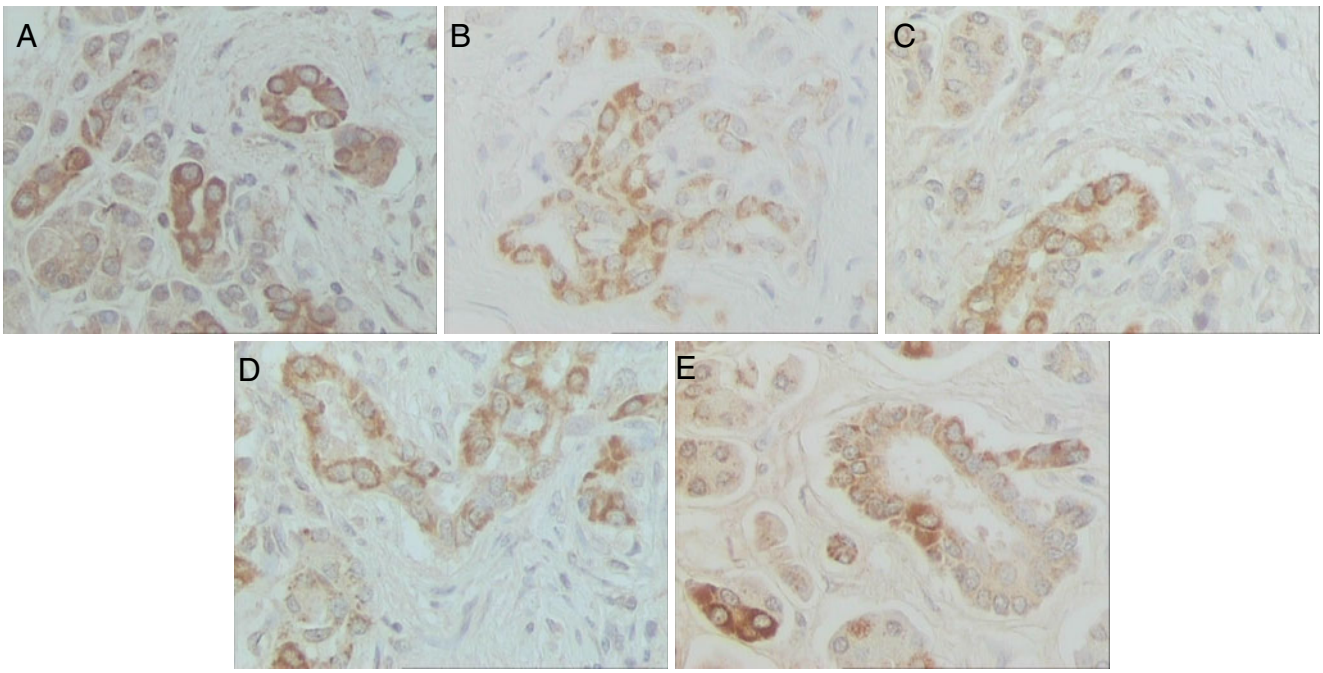

Fig. 1 Representative cases of cytoplasmic **a** Eph-A1, **b** Eph-A2, **c** Eph-A4, **d** Eph-A5 and **e** Eph-A7 expression in tumor cells of pancreatic adenocarcinoma: Streptavidin-biotin-peroxidase, DAB chromogen, Harris hematoxylin counterstain (original magnification X400)

Table 6 Spearman rank correlations amongst the Eph-A1, -A2, -A4, -A5 and -A7 percentage expression in the 67 pancreatic adenocarcinoma specimens

n=67	Eph-A1	Eph-A2	Eph-A4	Eph-A5	Eph-A7
Eph-A1	–	$p>0.05$	$p>0.05$	$p>0.05$	$p>0.05$
Eph-A2	$p>0.05$	–	$r_s=0.259$	$r_s=0.247$	$r_s=0.301$
			$p=0.034$	$p=0.044$	$p=0.013$
Eph-A4	$p>0.05$	$r_s=0.259$	–	$r_s=0.332$	$r_s=0.428$
		$p=0.034$		$p=0.006$	$p=0.0003$
Eph-A5	$p>0.05$	$r_s=0.247$	$r_s=0.332$	–	$r_s=0.321$
		$p=0.044$	$p=0.006$		$p=0.008$
Eph-A7	$p>0.05$	$r_s=0.301$	$r_s=0.428$	$r_s=0.321$	–
		$p=0.013$	$p=0.0003$	$p=0.008$	

creasing MMP-2 expression, in a FAK-dependent manner [21]. Importantly, Eph-A2 ligation by Ephrin A1-Fc inhibited cellular invasiveness, down-regulated total and cell surface levels of Eph-A2, and induced dephosphorylation of FAK and downregulation of MMP-2 expression. Thus, Eph-A2 may be considered as a rational target for therapeutic intervention in pancreatic neoplasia [21]. Furthermore, primary and metastatic pancreatic carcinomas presented significantly increased Eph-A2 immunoreactivity compared to benign ducts and pancreatic intraepithelial neoplasia lesions [22]. In addition, poorly differentiated carcinomas presented significantly increased Eph-A2 immunoreactivity compared to well and moderately differentiated tumors. Reduced Eph-A2 immunoreactivity was more commonly found in liver, lung or peritoneal metastases as compared to distant lymph node metastases. Genetic sequencing of the tyrosine kinase domain of EPHA2 in 22 samples of xenograft enriched pancreatic cancer did not reveal any inactivating mutations. However, EPHA2 amplification was found in 1 of 33 pancreatic cancers corresponding to lymph node metastasis, indicating that EPHA2 genomic amplification may result in EphA2 overexpression in a minority of patients. These data confirmed that Eph-A2 is overexpressed in pancreatic cancer, but suggested a relative loss of Eph-A2 in co-existent pancreatic cancer metastases as well as a role for EPHA2 in organ specific metastasis [22].

Our study verified that Eph-A1, -A2, -A4, -A5 and -A7 may play a role in pancreatic cancer biology, as they were abundantly expressed in approximately a half of pancreatic ductal adenocarcinoma specimens. In agreement with Mudali et al. [22], we showed that Eph-A1, -A2, -A4, -A5 and -A7 expression presented predominantly cytoplasmic staining. Eph-A1 was associated with tumor size, tumor histopathological stage and lymph node metastasis, but not with patients' survival. In this context, Abraham et al. showed that Eph-A1 was overexpressed in urothelial carcinoma compared to normal tissues; however, its levels were similar across all stages of bladder cancer [23]. In colorectal carcinomas, Eph-A1 expression was significantly associated with patients' gender, tumor histopathological grade and stage, depth of invasion and lymph node metastasis, as well as patients' survival [24, 25]. A correlation between high Eph-A1 expression and high

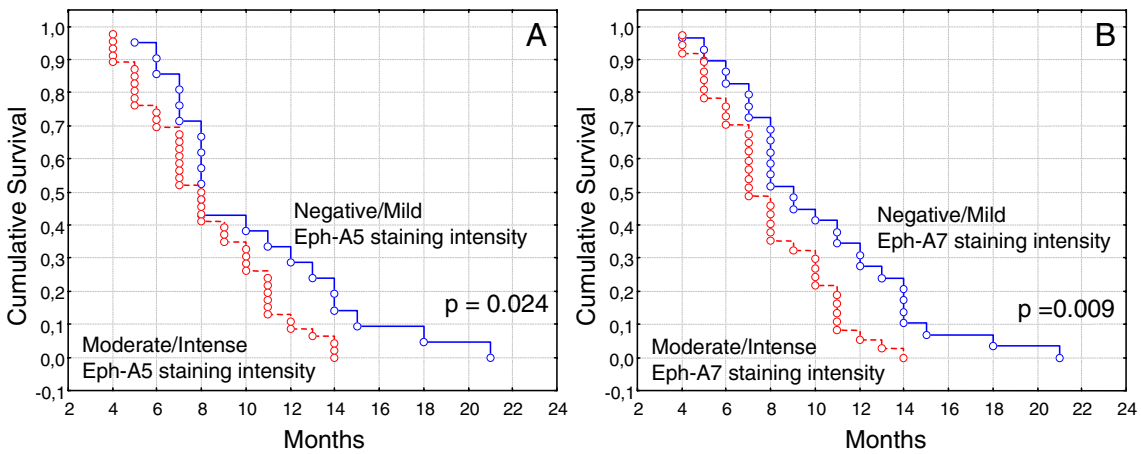

Fig. 2 Kaplan-Meier survival analysis stratified according to **a** Eph-A5 and **b** Eph-A7 staining intensity in patients with pancreatic adenocarcinoma. Negative/mild Eph-A5 and Eph-A7 staining intensity is depicted by blue continuous line, while moderate/intense Eph-A5 and Eph-A7 staining intensity is depicted by red dashed line

levels of cyclin A and p21, depth of invasion, advanced FIGO stage and poor survival was reported in vulvar carcinomas [26]. Hafner et al. also showed that Eph-A1 may represent a potential prognostic marker and therapeutic target in non-melanoma skin cancer [27].

Although the most comprehensive data so far demonstrated that Eph-A2 was associated with important clinicopathological characteristic for patients' management in a variety of malignant tumors [23, 26, 28–39], we did not find any clinical significance of Eph-A2 in pancreatic neoplasia except for a trend of correlation with patients' age. Moreover, Eph-A2 overexpression was significantly associated with poor prognosis in several types of malignant tumors, including that of oral tongue, as well as oesophageal, lung, cervical, ovarian, endometrial and renal carcinoma, as well as glioblastoma [26, 29, 33, 36, 38–41]. However, we did not find any significant association between Eph-A2 expression or staining intensity and patients' survival. In contrast, two other member of Eph receptor family, Eph-A5 and -A7, were identified for the first time as independent prognostic factors in pancreatic ductal adenocarcinoma. Among them, Eph-A7 overexpression was reported to be predictive of the adverse outcome in recurrent glioblastoma multiforme (GBM) patients [42].

In agreement with the present study, Iiizimi et al. revealed that Eph-A4 was overexpressed in approximately half of pancreatic ductal adenocarcinoma cases examined [43]. Interestingly, Iiizimi et al. further revealed that knockdown of Eph-A4 by siRNA drastically attenuated pancreatic ductal adenocarcinoma cell viability, while Eph-A4 introduction into pancreatic ductal adenocarcinoma cells lacking Eph-A4 expression resulted in growth promotion. Coexistence of eph-A3 ligand in pancreatic adenocarcinoma cells with Eph-A4 receptor and knockdown of eph-A3 ligand by siRNA also attenuated pancreatic ductal adenocarcinoma cell viability, suggesting that Eph-A4/eph-A3 pathway may be a promising target for pancreatic cancer therapy [43]. Oki et al. also showed that Eph-A4 overexpression was significantly associated with the depth of invasion and the recurrence of gastric cancer patients. Gastric cancer patients with EphA4-positive cancer presented significantly shorter overall survival times compared to those with EphA4-negative cancer [44]. In contrast, no significant associations between Eph-A4 and clinicopathological parameters, as well as patients' survival were noted in the present study.

In conclusion, the present report supported evidence that Eph-A1, -A2, -A4, -A5 and -A7 were frequently expressed in pancreatic adenocarcinoma tissue samples. Eph-A1 staining intensity was associated with important clinicopathological parameters for patients' management. Eph-A5 and -A7 were identified as significant prognostic factors. However, further molecular and clinical studies are required to delineate the potential clinical application of Eph receptors in prognosis and management of pancreatic adenocarcinoma patients and to elucidate whether Eph receptors could be considered as targets for therapeutic intervention in pancreatic neoplasia.

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