RESEARCH ARTICLE

Prognostic Impact of Cyclin D1, Cyclin E and P53 on Gastroenteropancreatic Neuroendocrine Tumours

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Abstract

Conventional classifications of gastroenteropancreatic neuroendocrine tumours (GEP- NETs) are rather unsatisfactory because of the variation in survival within each subgroup. Molecular markers are being found able to predict patient outcome in more and more tumours. The aim of this study was to characterize the expression of the proteins cyclin D1, cyclin E and P53 in GEP- NETs and assess any prognostic impact. Tumor specimens from 68 patients with a complete follow-up were studied immunohistochemically for cyclin D1, cyclin E and P53 expression. High cyclin D1 and cyclin E immunostaining (≥ 5% positive nuclei) was found in 48 (71%) and 24 (35%) cases, and high P53 staining (≥ 10% positive nuclei) in 33 (49%) . High expression of P53 was more common in gastric neuroendocrine tumors and related to malignant behavior, being associate with a worse prognosis on univariate analysis (RR=1.9, 95%CI=1.1-3.2). High expression of cyclin E was significantly associated with shorter survival in the univariate analysis (RR=2.0, 95%CI=1.2-3.6) and multivariate analysis (RR=2.1, 95%CI=1.1-4.0). We found no significant correlation between the expression of cyclin D1 and any clinicopathological variables. Our study indicated a prognostic relevance for cyclin E and P53 immunoreactivity. Cyclin E may be an independent prognostic factor from the 2010 WHO Classification which should be evaluated in further studies.

Keywords: Gastroenteropancreatic neuroendocrine tumor - immunohistochemistry - cyclin D1 - cyclin E - P53

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Introduction

GEP- NETs comprise a group of rare tumors arising from the neuroendocrine system of the gut. The estimated annual incidence is about 1-4 cases per 100 000 with an increasing trend of incidence over recent decades (Hemminki and Li, 2001; Quaedvlieg et al., 2001; Modlin et al., 2003; Lepage et al., 2004; Cho et al., 2012). GEP-NETs were classified traditionally according to their origin divisions of the gut (Williams and Sandler, 1963). However, the biological and clinical characteristics of the tumors vary greatly between the subgroups. More recently, the classification currently used is the new 2010 WHO classification of the endocrine tumors of the gastroenteropancreatic tract which is based on mitotic count and Ki67 index (Bosman and Carneiro, 2010). But the prognostic value of these new classifications still needs to be confirmed.

Nowadays, the molecular variables as useful predictors of malignant behavior have been investigated in more and more tumors. It is well known that tumor growth is the result of an uncontrolled cell proliferation or a defective cell death program. For the proliferation of the cells, the phosphorylation of pRb by Cyclin–cdk complexes to release the transcription factor E2F is an essential step. The Cyclin D1 and CDK4 or CDK6, Cyclin E and CDK2 composed of the Cyclin–CDK complexes. The absence or functional abnormality of P53 allows tumor to progress uncontrolly and escape from apoptosis. Previously, overexpressions of Cyclin D1, E and P53 proteins have been demonstrated in many tumors and correlated with prognosis (Florenes et al., 2000; Richter et al., 2000; Kamai et al., 2001; Heah et al., 2011). However, insights into deregulation of apoptosis and cell cycle in GEP-NETs with respect to the clinical outcome are scarce.

To elucidate further the molecular pathogenesis of GEP-NETs and to identify molecular markers predictive of patient's outcomes, we tested the expression of the Cyclin D1, Cyclin E, and P53 in a series of patients with GEP-NETs.

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Materials and Methods

Patients

A retrospective survey of 68 patients with neuroendocrine tumors of the pancreas and gastrointestinal tract, undergoing surgery at Henan Cancer Hospital in the period 2000-2010, was performed. No chemotherapy or radiotherapy was given before surgery. All patients were followed until November 30, 2012.

The study was approved by the ethnics committee of Henan Cancer Hospital. Tumors were graded according to the 2010 Who Classification and 9 (13.2%) were G1 (ki67≤2%), 37(54.4%) were G2 (3%-20%) and 22 (32.4%) were G3 (>20%).

Immunohistochemistry

Sections for immunohistochemistry were stained using the avidin–biotin complex (ABC) method. 5um thick sections were cut from the blocks, deparaffinized with xylene and dehydrated through graded concentrations of alcohol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min. The sections were then treated with microwave radiation for 10 min for antigen retrieval, and, to block intrinsic antibody binding, they were reacted with normal serum (mouse IgG) for 10 min at room temperature. The sections were then incubated with primary antibodies Cyclin D1 (1:10 dilution, Clone DSC-6; PROGEN, Heidelberg, Germany), Cyclin E(1:50 dilution, clone HE12, BD Pharmingen, San Diego, CA) and P53 (1:50 dilution, Clone PAb 1801, BD Pharmingen, San Diego, CA) overnight at 4°C, with appropriate negative and positive controls, they were reacted with biotinylated anti-mouse antibody (secondary antibody) for 10 min and with ABC for another 10 min, with intervening washes. Diamino- benzidine tetrahydrochloride was used as the final chromogen, and sections were counterstained with Mayer's haematoxylin before mounting.

Five semi-quantitative classes were used to describe the number of positively stained tumor cells; none, <5% of the cells, <10% of the cells, 10–50% of the cells, and >50% of the cells. Protein levels were classified as high when any ≥5% of cells were positive for Cyclin D1 and Cyclin E, and ≥10% of cells were positive for P53 according to the previous studies (Sakaguchi et al., 1998; Sumiyoshi et al., 2006; Ioachim 2008; Kishimoto et al., 2008). All slides were evaluated the same day by two pathologists to minimize the variability of the result.

Statistical analyses

Fisher exact test was used to compare the distribution of various patient characteristics by expression of Cyclin D1, Cyclin E and P53. Survival rates were calculated by the Kaplan Meier method. Univariate and multivariate relative risks (RRs) of dying were calculated using Cox proportional hazards regression. Patients were censored after 5 follow-up years or November 30, 2012. In the multivariate analysis, forward stepwise regression with $P = 0.10$ as inclusion criteria was used. Cyclin D1, Cyclin E and P53, however, were included in all the multivariate models. All calculations were performed using the SPSS 11.0 statistical software package. A significance level of 0.05 was chosen.

Results

The immunohistochemical results in GEP-NETs are summarized in Table 1. For Cyclin D1, low (<5% positive nuclei) and high (≥5% positive nuclei) protein levels were detected in 20 (29%) and 48 (71%) tumors, respectively. Low Cyclin E immunoreactivity (<5% positive nuclei) was found in 44 (65%) tumors, whereas high (≥5% positive nuclei) was seen in 24 (35%) of the cases. Low (<10% positive nuclei) and high (≥10% positive nuclei) P53 was found in 35 (51%) and 33 (49%) cases, respectively.

Table 2 shows the immunohistochemical expression of the Cyclin D1, Cyclin E and P53 in relation to clinicopathological variables. The expression of Cyclin D1 and Cyclin E did not correlate with any of the clinicopathological variables. P53 was significantly correlated to tumor origin ($P<0.01$) and WHO Classification ($P=0.03$).

Examples for immunohistochemical staining of Cyclin D1, Cyclin E and P53 are given in Figure 1.

Table 1. Immunostaining Results for P53, P21 and P27

Expression	Number of cases		
	Cyclin D(%)	Cyclin E(%)	P53(%)
Negative	15(22)	40(59)	24(35)
<5%	5(7)	4(6)	3(4)
<10%	2(3)	5(7)	8(12)
10-50%	12(18)	16(24)	25(37)
>50%	34(50)	3(4)	8(12)

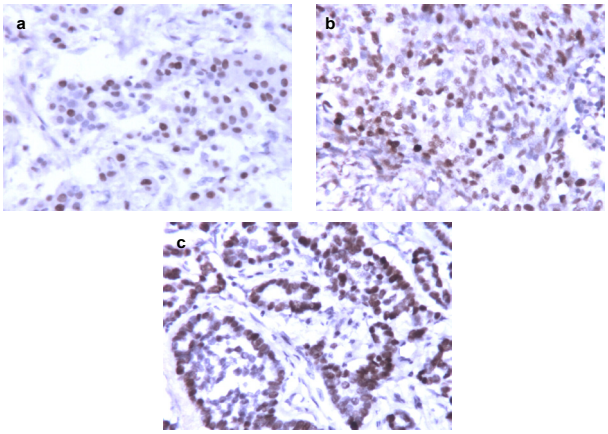

Figure 1. Immunohistochemical Analysis Showing High Cyclin D1(a), Cyclin E(b), and P53(c) Protein Expression

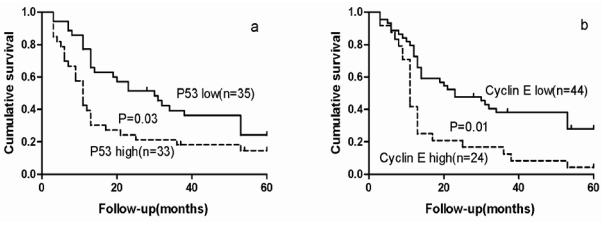

Figure 2. Kaplan-Meier Survival Curves for Patients of GEP-NETs for High and Low Expression of P53(a) and Cyclin E(b)

Table 2. Patient's Demographics and Clinical Features

variable	Total	Cyclin D expression			Cyclin E expression			P53 expression		
	N	Low	High(%)	P^a	Low	High(%)	P^a	Low	High(%)	P^a
sex				1.00			1.00			0.27
Male	51	15	36(71)		33	18(35)		24	27(53)	
Female	17	5	12(71)		11	6(35)		11	6(35)	
Age(y)				0.32			0.69			0.64
<60	38	11	27(71)		26	12(32)		21	17(45)	
60-69	13	2	11(85)		7	6(46)		7	6(46)	
≥70	17	7	10(59)		11	6(35)		7	10(59)	
Tumor origin				0.44			0.26			0.00
Gastric	51	13	38(75)		30	21(41)		20	31(61)	
Colon and Rectum	10	4	6(60)		8	2(20)		9	1(10)	
Pancreas	7	3	4(57)		6	1(14)		6	1(14)	
WHO Classification				0.75			0.40			0.03
G1	9	3	6(67)		7	2(22)		8	1(11)	
G2	37	12	25(68)		21	16(43)		19	18(49)	
G3	22	5	17(77)		16	6(27)		8	14(64)	
Stage				0.77			0.09			0.13
Confined within organ	28	7	21(75)		20	8(29)		18	10(36)	
Invasion of adjacent organ	5	2	3(60)		5	0(0)		1	4(80)	
Distant metastasis	35	11	24(69)		19	16(46)		16	19(54)	
Tumor size(cm)				1.00			0.73			0.51
<2	11	3	8(73)		8	3(27)		7	4(36)	
>2	57	17	40(70)		36	21(37)		28	29(51)	
Localization of the metastases				0.69			0.21			0.42
Liver	8	3	5(63)		7	1(13)		5	3(38)	
Other	27	8	19(70)		15	12(44)		11	16(59)	

[a]Fisher exact test

Table 3. Relative Risks(RR) of Dying from Gastroenteropancreatic Neuroendocrine Tumors

Variable	Univariate analysis			Multivariate analysis		
	RR	95%CI	*P*	RR	95%CI	*P*
sex			0.13			
Male	1	-				
Female	1.7	0.9-3.4				
Age(y)						
<60	1	-	0.71			
60-69	1.3	0.7-2.6				
≥70	1.5	0.8-2.8				
Tumor origin			0.05			
Gastric	1	-				
Colon and Rectum	0.7	0.3-1.5				
Pancreas	0.1	0-0.5				
WHO Classification			<0.01			<0.01
G1	1	-		1	-	
G2	13.5	1.8-99		11.1	1.5-83.5	
G3	25.5	3.4-193		24.4	3.2-188.7	
Stage			<0.01			<0.01
Confined within organ	1	-		1	-	
Invasion of adjacent organ	1.3	0.7-2.2		1.1	0.6-2.2	
Distant metastasis	5.3	2.7-10.3		5.0	2.5-9.9	
Tumor size(cm)			0.08			
<2	1	-				
>2	2.2	0.9-5.1				
localization of the metastases				0.41		
Liver	1	-				
Other	0.7	0.3-1.6				
Cyclin D1 expression			0.32			0.56
<5%(low)	1	-		1	-	
>5%(high)	1.4	0.7-2.5		1.2	0.6-2.4	
Cyclin E expression			0.01			0.02
<5%(low)	1	-		1	-	
>5%(high)	2.0	1.2-3.6		2.1	1.1-4.0	
P53 expression			0.03			0.31
<10%(low)	1	-		1	-	
>10%(high)	1.9	1.1-3.2		1.3	0.8-2.3	

The associations between clinicopathological, immunohistochemical data and survival in univariate and multivariate analyses are presented in Table 3. The result from the univariate analysis for P53 and Cyclin E is presented in a Kaplan Meier survival curve in Figure 2. High expression of P53 and Cyclin E indicated prognostic relevance with a RR of 1.9 (95% CI: 1.1-3.2) and 2.0 (95% CI: 1.2-3.6) separately, revealing a worse prognosis for patients presenting with tumors expressing high levels of P53 or Cyclin E. In the multivariate analysis WHO Classification, stage and Cyclin E was the only parameters with statistical significance.

Discussion

In the present study, we found no prognostic significance for Cyclin D1. This is in accordance with previous studies of GEP-NETs. Kawahara found Cyclin D1 protein was detected in most of the tumors, 100% of the malignant group and 94.3% of the benign group, and no correlation with malignant behavior (Kawahara et al., 2002).

Overexpression of Cyclin E has been extensively documented in carcinomas of the gastric (Ahn et al., 1998) and colon (Corin et al., 2010). Our study revealed high Cyclin E protein expression in 35% of the cases. This is in agreement with the study of patricia (Grabowski et al., 2008), identifying Cyclin E overexpression in 38 of 89 (43%) GEP-NETs.

A high level of Cyclin E expression was shown to be associated with decreased overall survival in univariate and multivariate analysis. Our results demonstrated that Cyclin E may be an independent indicator of survival in

GEP-NETs from the WHO Classification.

We found high expression of P53 in 49% of GEP-NETs. Usually p53 positivity by immunohistochemistry indicates a mutated form of the protein, although other cellular aberrations theoretically may result in overexpression of p53 (Rorstad, 2005). The high expression of P53 was more common in gastric endocrine tumors and positive correlated with the WHO Classification. Our finding suggested that overexpression of p53 appeared to confer a more malignant prognosis.

P53 immunoreactivity was found to be a predictor of survival in univariate analyses but not a predictor in multivariate analyses. This may be explained by the positive correlation between P53 and WHO Classification. The Ki-67 proliferation index has been proposed in the WHO classification (G1, G2, G3). The index refers to the percentage of cells, which are positive by immunohistochemistry for this antigen in a tumor section.

In conclusion, our study demonstrates a prognostic relevance for P53 and Cyclin E proteins. High level of P53 protein and Cyclin E protein were correlated to a shorter disease-related survival. We did not, however, find Cyclin D1 protein expression to be useful as a prognostic indicator in GEP-NETs. And high Cyclin E expression may be an independent prognostic factor from the WHO Classification which should be evaluated in further studies. We therefore propose to incorporate the immunohistochemical expression of Cyclin E into a new classification to individualize therapeutic stategies in the future.

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