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Original Article

Role of Hepatocyte Paraffin 1 antigen in the course of colorectal carcinogenesis

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Abstract: Background: Few studies have reported the expression of Hepatocyte Paraffin 1 (Hep Par 1) in colorectal carcinomas with contradictory results. Reported rate of expression ranged from 4-50%. Moreover, the correlation between Hep Par 1 expression and clinicopathological parameters has not been investigated. The objective of the present study was to investigate the role of CPS1 (using Hep Par 1) in colonic carcinogenesis and characterize carcinomas which express it. Material and Method: Comparative analysis was done between Hep Par 1 expression in normal colonic mucosa (n=10), adenomatous polyps (n=29) and sporadic adenocarcinoma (n=40) and was correlated with clinicopathologic parameters. Results: Normal colonic mucosa did not express Hep Par 1. In contrast, it was expressed in dysplastic glands and neoplastic cells of well-moderately differentiated non-mucinous adenocarcinomas. Hep Par 1 was found in 47.5% of colonic carcinomas, 41.7% of polyps with high grade dysplasia (HGD) and 23.5% of polyps with low grade dysplasia (LGD). Mean Hep Par-1 score, likewise, was highest in carcinoma, high in polyps with HGD and lowest in polyps with LGD. Hep Par 1 expression inversely correlated with some conventional prognostic parameters including tumour type, grade, lymph node metastasis and AJCC stage. It did not correlate with depth of invasion or lymphovascular invasion. Conclusion: Hep Par 1 (i.e. CPS1) might play an active role in initiation of dysplasia and progression of multistep colorectal carcinogenesis. However, it seems that CPS1 is not involved in invasion and tumour spread. Conversely, it might be in the play of suppressing cancer progression. These findings could have both prognostic and therapeutic applications.

Keywords: Adenomatous polyp, colonic carcinoma, Hep Par 1, progression

Introduction

The monoclonal antibody Hepatocyte Paraffin 1 (Hep Par 1) identifies an intra-mitochondrial epitope carbamoyl phosphate synthetase 1 (CPS1) [1] which is a rate-limiting enzyme of the urea cycle [2]. It was thought that the liver is the mere organ responsible for urea synthesis. Thus, Hep Par 1 was widely used to discriminate hepatocellular carcinoma from intrahepatic cholangiocarcinoma and metastatic carcinomas [3, 4]. Recently, Hep Par 1 was found in normal small intestinal epithelium [2, 5, 6], intestinal metaplasia in Barrett oesophagus and chronic gastritis [6]. Also, it was expressed in adenocarcinomas of the stomach, ovary, adrenal cortex, lung, endocervix, pancreas, breast and neuroendocrine carcinomas [5, 7-9]. These findings have ignited the curiosity about its physiologic and pathogenetic role.

Although Hep Par 1 was found to be expressed in normal small intestinal epithelium, it was frequently lost in small intestinal adenocarcinoma [10]. In contrast, it was not expressed in normal colonic mucosa and was acquired in some colonic adenocarcinomas [10]. These observations raised questions about its (or CPS1) role in intestinal tumorigenesis. Quite few literatures have reported the expression of Hep Par 1 in colorectal carcinomas. The results were contradictory with a reported rate of expression ranging from 4-10% [5, 10] up to 50% [7, 11]. Interestingly, a recent preliminary study found Hep Par 1 expression in colorectal polyps with dysplasia [11]. These findings warranted additional investigations.

The objective of the present study was to investigate the role of CPS1 (using Hep Par 1 antibody) in colonic carcinogenesis. Thus, a com-

parative analysis was done between the expression of Hep Par 1 in colonic adenomatous polyps and carcinomas. Furthermore, aiming to characterize tumours which acquire Hep Par 1 expression and to attest its possible role in the biological behaviour of colonic carcinomas, this study, for the first time, correlated the expression of Hep Par 1 with different clinicopathologic parameters.

Material and methods

Seventy-nine consecutive colorectal specimens retrieved from the archives of Ain Shams University Hospitals and Ain Shams Specialized Hospital were studied retrospectively. To evaluate the role of Hep Par 1 in multistep carcinogenesis, comparison was done between normal colonic mucosa (n=10), adenomatous polyps (n=29) and sporadic colorectal adenocarcinomas (n=40). Normal colonic mucosa specimens were taken from the margins of colectomy specimens done for reasons other than carcinomas (e.g. intestinal obstruction). Cases of serrated adenomas, familial adenomatous polyposis or inflammatory bowel disease were excluded. Patients' data were obtained from the reports including age, sex, tumour size, presence of nodal and distant metastasis. Patients in the study cohort did not receive preoperative adjuvant chemotherapy and/or radiotherapy.

Standard histopathological examination and classification were done. Adenomatous polyps were classified into low and high grade dysplasia. The colorectal adenocarcinomas were evaluated in accordance to World Health Organization (WHO) classification [12] with emphasis on histological variant, grade, depth of invasion, lymphovascular invasion and lymph node status. Tumours were staged in accordance with the (TNM) classification of malignant tumours in AJCC Cancer Staging Manual, 7th edition [13].

Immunohistochemistry

The primary antibody Hep Par 1 (Dako, clone OCH1E5, 1:50 dilution, Carpinteria, CA, USA) was used. Paraffin embedded tissue sections (5-μm thick) were deparaffinised in xylene and rehydrated through absolute alcohol. Antigen retrieval in citrate buffer (pH 9) was used after the sections were treated in a microwave at 8 W for 5-6 min, then at 3 W for 10 minutes. Sections were left to cool for 20 minutes. Peroxidase block and protein block were done. Slides were incubated with the primary antibody at room temperature for one hour followed by rinsing in PBS (pH 7.6). This was followed by the secondary biotin conjugated antibody for 1 hour and finally the peroxidase conjugated streptavidin for another hour. Diaminobenzidine tetrachloride (DAB) (freshly prepared) was added for 25 minutes, then counter staining in Harris haematoxylin, followed by dehydration, clearing and mounting. Sections of human liver needle biopsies (n=2) were used as an appropriate positive control. Negative controls were performed by omitting the primary antibody.

Evaluation of Hep Par 1 expression

A case was considered positive if 5% or more of cells of interest showed cytoplasmic staining. Analysis was done using computerized Image Analyzing Software (Special FIF starter. version 3.2, Olympus, Germany) connected to an Olympus microscope (model BX51, Olympus Japan). Evaluation of Hep Par 1 expression was adopted from Mac et al. and Ramos-varaet et al. [10, 14]. The mean percentage of immunostaining was assessed by counting positive and negative cells in five high power fields (x400). Staining extent was scored as 0 (<5%), 1 (5-10%), 2 (11-50%), 3 (51-80%) and 4 (>80%). Sections were scored independently by two pathologists to avoid inter-observer variability. Inter-observer agreement was 90%. Disagreement between readings was resolved in consequence through re-examining the cases by both pathologists.

Statistical analysis

The collected data was revised, coded, tabulated and introduced to a PC using Statistical package for Social Science (SPSS 15.0.1 for windows; SPSS Inc, Chicago, IL, 2001). Continuous variables were expressed as mean and standard deviation (±SD). Categorical variables were expressed as frequencies and percents. Differences between independent groups were tested using the student *t* test for continuous variables. Categorical variables were compared using the chi-square test or Fisher exact test. Kruskal-Wallis test was used to compare an ordinal variable between more than two study groups. Mann Whitney test were used to com-

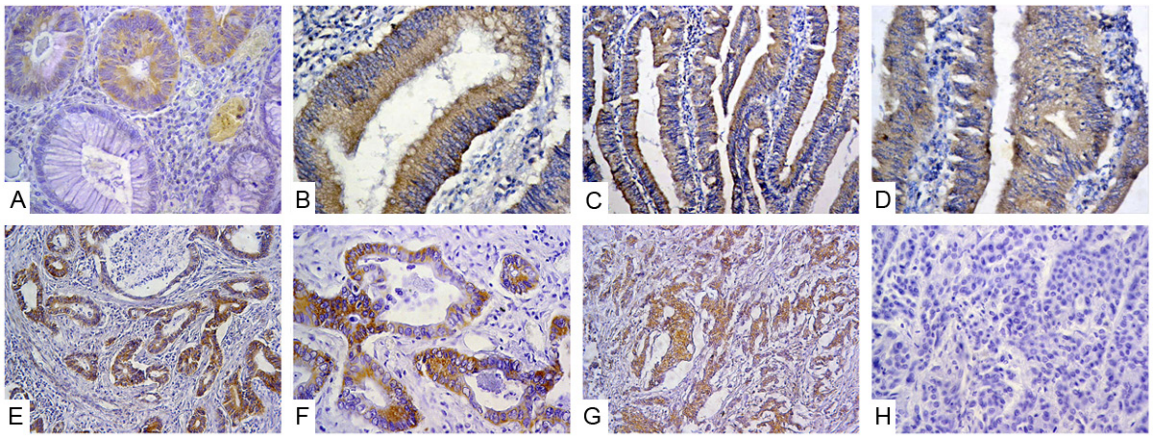

Figure 1. Hepatocyte Paraffin 1 (Hep Par 1) expression in polyps and adenocarcinomas. Positive cytoplasmic expression was seen in: A: Adenomatous polyp with low grade dysplasia in dysplastic glands while adjacent hyperplastic glands are negative, B: adenomatous polyp with low grade dysplasia in dysplastic glands, C&D: adenomatous polyp with high grade dysplasia, E&F: well differentiated adenocarcinoma and G: moderately differentiated adenocarcinoma. Negative Hep Par 1 expression was seen in: H: Poorly differentiated carcinoma. (Immunohistochemistry, original magnification A, B, D, F, H x400; C, E, G x200).

pare an ordinal variable between two study groups. *P*-Value (level of significance) was assigned >0.05 as non-significant (NS), <0.05 as Significant (S) and <0.01 as highly significant (HS).

Results

Clinicopathologic results

The 29 cases of adenomatous colonic polyps included 13 (44.8%) men and 16 (55.2%) women with ages ranging from 27 to 73 years (mean±SD, 47±12 years). The 40 cases of colonic adenocarcinomas included 23 (57.5%) men and 17 (42.5%) women with ages ranging from 29 to 77 years (mean±SD, 51.5±15 years). There was no statistical difference between the two groups regarding age and gender. Tumour size of colonic carcinomas ranged from 2 to 10 cm (mean±SD, 5.76±1.99 cm).

The adenomatous polyps were categorized into 17 cases (58.6%) with low grade dysplasia (LGD) and 12 cases (41.4%) with high grade dysplasia (HGD). In colonic carcinomas, 34 cases (85%) were adenocarcinomas (NOS) and 6 cases (15%) were mucinous adenocarcinoma. Well-differentiated, moderately differentiated and poorly differentiated adenocarcinomas constituted 32.5%, 40% and 27.5%, respectively. Five cases invaded the submucosa (pT1) (12.5%), twelve cases invaded the muscularis propria (pT2) (30%), twenty-one cases invaded through muscularis propria into the pericolorectal tissue (pT3) (52.5%) and two cases penetrated to the surface of visceral peritoneum (pT4) (5%). Lymphovascular invasion was present in 5 cases (12.5%). Lymph node metastases were present in 25 cases (62.5%). Tumours were categorized into stage I (n=9, 22.5%), IIa (n=6, 15%), IIIA (n=6, 15%), IIIB (n=7, 17.5%) and IIIC (n=12, 30%).

Immunohistochemical results

Diffuse granular cytoplasmic staining was observed in liver biopsies included as a positive control. In normal colonic mucosa (n=10), Hep Par 1 was completely immunonegative. Focal to diffuse Hep Par 1 expression was noted in 9 (31%) of 29 adenomatous polyps and in 19 (47.5%) of 40 carcinomas. Positivity was evident in the dysplastic glands of the adenomatous polyps and in the neoplastic cells of the well differentiated and moderately differentiated adenocarcinomas. Poorly differentiated adenocarcinoma and mucinous adenocarcinoma did not express Hep Par 1. Representative photomicrographs of Hep par 1 in adenomatous polyps and adenocarcinomas are demonstrated in **Figure 1**.

Hep par 1 expression in normal, adenomatous polyps and neoplastic colorectal tissues

Hep Par 1 expression was more frequently expressed in colorectal carcinoma (47.5%)

Table 1. Expression of Hep Par 1 in normal colonic mucosa, adenomatous polyps and carcinoma (n=79)

Tissue sample	n	Hep Par 1		*P* value
		Negative (n=51)	Positive (n=)	
Normal mucosa	10	10 (100%)	0 (0%)	0.0179[a]
Adenoma with LGD	17	13 (76.5%)	4 (23.5%)	
Adenoma with HGD	12	7 (58.3%)	5 (41.7%)	
Carcinoma	40	21 (52.5%)	19 (47.5%)	

Abbreviations: LGD, low grade dysplasia. HGD, high grade dysplasia. [a]Fisher's exact Test. *P*-value presents the differences between the four groups.

Table 2. Hep Par 1 score in adenomatous polyps and carcinoma

Tissue sample	n	Score		*P* value[a]
		Mean±SD	Median	
Adenoma with LGD	17	1.5±0.6	1.5	
Adenoma with HGD	12	2.8±0.4	3	0.01[a]
Carcinoma	40	2.9±0.7	3	

Abbreviations: LGD, low grade dysplasia. HGD, high grade dysplasia. [a]*P*-value presents the differences between the three groups, Kruskalwallis test. *P* value of carcinoma versus adenoma with LGD=0.004, Mann Whitney test. *P* value of carcinoma versus adenoma with HGD=0.688, Mann Whitney test. *P* value of adenoma with HGD versus LGD=0.018, Mann Whitney test.

compared to HGD (41.7%) and LGD (23.5%), (p=0.017) (**Table 1**). Mean Hep Par 1 score was significantly highest in colorectal carcinoma, high in HGD and lowest in LGD (p=0.01). Comparing each two groups individually, Hep Par 1 mean score was significantly higher in carcinoma compared to LGD (p=0.004) and in HGD compared to LGD (p=0.018). No significant difference was observed between HGD and carcinomas (p=0.68) (**Table 2**).

Correlation between Hep Par 1 expression and clinicopathologic characteristics in colorectal adenocarcinomas

There was no correlation between the Hep Par 1 expression and age, gender or tumour size. Hep Par 1 was more frequently expressed in non-mucinous (p=0.021) adenocarcinomas, better (well and moderate) differentiated tumours (p=0.0001), without nodal spread (p=0.003) and with low AJCC tumour stage (p=0.019). Conversely, Hep Par 1 was not expressed in all poorly differentiated adenocarcinomas, mucinous adenocarcinomas and tumours with lymphovascular invasion. Likewise, mean Hep Par 1 score was significantly higher in well and moderately differentiated versus poorly differentiated adenocarcinomas (p=0.001), non-mucinous versus mucinous adenocarcinomas (p=0.03), absence versus presence of lymph node metastasis (p=0.002) and lower versus higher AJCC tumour stage (p=0.011). No significant correlation was found between Hep Par 1 expression and depth of invasion or lymphovascular invasion (**Table 3**).

Discussion

Hep Par 1 is widely recognized in surgical pathology as a relatively sensitive and specific marker of hepatocellular differentiation [1]. CPS1, the antigen of Hep Par 1, was considered to exist solely in hepatocytes where the conversion of ammonia to urea occurs [1]. Thus, in parallel with Mac et al. [10] and Nemolato et al. [11], the normal colonic mucosa in this study did not express Hep Par 1.

In the present study, Hep Par 1 expression was detected in 23.5% of adenomatous polyps with low grade dysplasia (LGD) and 41.7% of adenomatous polyps with high grade dysplasia (HGD). This supports a recent preliminary study of Nemolato et al. [11] that found Hep Par 1 in 50% of adenomatous polyps with HGD. Moreover, in agreement with previous studies [7, 11], 47.5% of colonic adenocarcinomas, in this study, expressed Hep Par 1. Its absence in normal mucosa, on one hand, and its presence in adenomatous polyps and adenocarcinoma on the other hand suggest that Hep Par 1 (i.e. CPS1) might share in the initiation of dysplasia-carcinoma sequence. The mean Hep Par 1 score, likewise, significantly increased from 1.5 (±0.6) in polyps with LGD, to 2.8 (±0.4) in HGD to 2.9 (±0.7) in carcinoma. This gradual increase signifies that CPS1 might have a potential role in the progression of multistep process of carcinogenesis.

A recent study suggested the exploitation of CPS1 for the future development of biomarkers to predict dysplasia in ulcerative colitis [15]. In the current study, comparing each two groups individually, Hep Par 1 expression significantly

Table 3. Correlation between Hep Par 1 expression and clinicopathological parameters in colonic carcinomas (n=40)

Parameter		Total	Hep Par 1			Hep Par 1 score	
		n	Negative n=21	Positive n=19	*P* value	Mean±S.D	*P* value
Age	<51.5	18	-	-	0.214[a]	1.77±1.39	0.229[d]
	≥51.5	22	-	-		1.09±1.65	
Sex	Male	23	11	12	0.491[b]	1.47±1.56	0.766[d]
	Female	17	10	7		1.29±1.61	
Size	<5.2 cm	21	10	11	0.5169[b]	1.42±1.46	0.936[d]
	≥5.2 cm	19	11	8		1.36±1.7	
Tumour type	Non-mucinous	34	15	19	**0.021**[c]	0±0.00	**0.03**[d]
	Mucinous	6	6	0		1.6±1.57	
Differentiation (grade)	Well	13	4	9	**0.0001**[b]	2.23±1.64	**0.001**[e]
	Moderate	16	6	10		1.68±1.44	
	Poor	11	11	0		0±0.00	
T category (Depth)	pT1	5	1	4	0.167[c]	2.40±1.67	0.176[e]
	pT2	12	5	7		1.75±1.60	
	pT3	21	13	8		1.10±1.48	
	pT4	2	2	0		00±00	
N category	N0	15	3	12	**0.003**[b]	2.47±1.46	**0.002**[e]
	N1	13	8	5		1.15±1.52	
	N2	12	10	2		0.33±0.78	
AJCC Stage	I	9	2	7	**0.019**[c]	2.56±1.59	**0.011**[e]
	IIA	6	1	5		2.33±1.37	
	IIIA	6	4	2		1.00±1.55	
	IIIB	7	4	3		1.29±1.60	
	IIIC	12	10	2		0.33±0.78	
Lymphovascular invasion	Absent	35	16	19	**0.048**[c]	1.6±1.57	0.05[d]
	Present	5	5	0		0±0.00	

[a]Student T-test. [b]Chi-Square test. [c]Fisher exact test. [d]Mann Whitney test. [e]Kruskalwallis test. P<0.05 are highlighted in bold. Age and tumour size were dichotomized by the median value.

varied in polyps with LGD compared to polyps with HGD or compared to carcinoma. On the other hand, no significant variation was observed between polyps demonstrating HGD and carcinoma. It is possible that CPS1 alteration might be a surrogate marker to reflect the progression of adenomatous polyps with LGD to HGD and/or carcinoma. Consequently, regulating CPS1 gene expression might be a potential target for treatment of these patients. Hep Par 1 shares in the metabolism of amino acid with production of nitric oxide [16]. This nitric oxide might serve in the evolution of carcinoma from adenomatous polyps. Nitric oxide has been implicated in the processes of tumour initiation, promotion and progression [17]. For example in chronic colonic inflammation, increased production of nitric oxide contributes to tumour progression through selecting cells with oncogenic mutant β-catenin regulatory genes [18]. It can directly damage DNA, inhibit DNA repair, enhance oncogene expression, modulate transcriptional factors, block apoptosis and contribute to angiogenesis. Moreover, it may stimulate COX-2 activity enhancing tumour promoting prostaglandins, e.g. Peroxynitrite and Cyclopentenone, which inactivate p53 and suppress tumour cell apoptosis [17, 19]. Identifying the possible underlying molecular mechanism of CPS1 in colorectal carcinogenesis might be a valuable future line of investigation.

The current study, for the first time, analysed the association of Hep Par 1 expression with clinicopathologic features in colorectal carcino-

mas. Hep Par 1 expression inversely correlated with some conventional prognostic parameters including tumour type, grade, lymph node metastasis and AJCC stage. It was absent in poorly differentiated adenocarcinomas and in mucinous adenocarcinomas. Its expression tended to decrease with nodal spread and higher AJCC tumour stage. These findings suggested that CPS1 might be lost in biologically aggressive colorectal cancers. In well and moderately differentiated adenocarcinomas Hep Par 1 was frequently expressed with a high mean score. This association with tumour grade is similar to the liver where it is expressed in differentiated hepatocellular carcinoma but not in poorly differentiated tumours [2, 20]. This was also demonstrated in gastric carcinomas [21]. CPS1 might be a function of differentiation with tendency to negativity in high-grade tumours [22]. Hep Par 1 expression neither correlated with the depth of tumour invasion nor with lymphovascular invasion. Moreover, it showed propensity to decline with nodal spread. This could indicate that CPS1 is not involved in invasion and spread of colonic carcinomas. All the aforementioned findings imply that CPS1 might be in the play of suppressing cancer progression. This is in accordance with the study of Fan et al [23] in gastric carcinoma. However, it is indistinct whether biologically aggressive colonic adenocarcinomas were natively negative to Hep Par 1 or did they lose Hep Par 1 expression during their evolution process from indolent to aggressive tumours. The functional significance of CPS1 in cancer cell behaviour warrants further investigations. However, for the time being, high positive expression could designate indolent tumours.

The positivity of Hep Par 1 expression in colonic carcinomas, demonstrated in the present study, reduces the high specificity of Hep Par 1 as a diagnostic marker for hepatocellular carcinoma. Nevertheless, this is not applicable to poorly differentiated tumours, tumours with high nodal status or high stage as it is more likely to be negative in these tumours. What makes the problem more complicated is that also poorly differentiated HCC tend to be negative for Hep par 1 expression [1, 20]. This emphasizes that Hep par 1 should be cautiously employed in differentiating primary HCC from metastatic colonic carcinoma in the liver.

Although the results of this study are promising, the study is limited by the small number of cases. This was done as a pilot study to characterize tumors which express Hep Par 1. It paves the way for future studies to look into the pathophysiological role and the regulatory mechanisms of CPS1 gene expression in three stratified groups; namely 1) adenomatous polyps with LGD, 2) HGD and well-moderate differentiated non-mucinous adenocarcinomas, pNo, low AJCC stage and 3) other colorectal carcinomas.

In summary, this comparative analysis provided evidence for an active role of Hep Par 1 (i.e. CPS1) in initiation of dysplasia and progression of multistep colorectal carcinogenesis. To the best of our knowledge; this is the first report to correlate Hep Par 1 over-expression in colonic carcinomas with favourable prognostic factors. It is over-expressed in well-moderate differentiated non-mucinous adenocarcinomas, without nodal spread and with low AJCC stage. CPS1, although involved in cancer cell development and differentiation, is not involved in invasion and tumour spread. Conversely, it might be in the play of suppressing cancer progression. These findings could have both prognostic and therapeutic applications. Further investigations on a larger scale are warranted.

Disclosure of conflict of interest

None.

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