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RAGE EXPRESSION in HCV-RELATED HEPATOCELLULAR CARCINOMA; COMPARISON BETWEEN MALIGNANT AND PERIMALIGNANT TISSUES

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ABSTRACT

Background: The expression of the receptor for advanced glycation end products (RAGE) has an impact on the mechanisms giving rise to characteristic features of various cancer cells. We aimed in this study to correlate the scores of RAGE expression with histopathological features of HCV-related hepatocellular carcinoma in malignant and peri-malignant tissue specimens.
Methods: The expression of RAGE was assessed in paired cancer and peri-malignant tissues of HCC, using immunohistochemistry. The expression scores were analyzed in association with the histopathological parameters of hepatitis and HCC.
Results: The expression of RAGE was less in hepatitis than in dysplasia and HCC. Furthermore, in HCC the expression increased initially with increased grade of malignancy and with increased intra-tumorous inflammatory infiltration, however, the score of RAGE expression decreased in high grades of malignancy.
Conclusions: Our results suggest that during the early stage of tumorigenesis with less blood supply HCC may acquire resistance to stringent hypoxic milieu by hypoxia-induced RAGE expression and then decreased. So we can select early cases with dysplasia and low grade malignancy as targets for future therapy.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most commonly diagnosed malignant disease and the second most common cause of cancer death worldwide, especially in Asia and Africa (El-Serag, 2007). Most cases of HCC are inflammation-related malignancies, which develop as a consequence of underlying liver disease, mostly viral hepatitis. The receptor for advanced glycation end products (RAGE) is a multiligand receptor classified as an immunoglobulin superfamily cell-surface molecule (Vlassara *et al*., 1989) and is recognized to be responsible for cancer progression in several human cancers. RAGE in HCC may be a novel, independent biochemical marker with the capacity to predict therapeutic outcome and may also be a new therapeutic target for patients with HCC after hepatectomy. (Ito *et al*., 2014) The prognosis of patients with HCC still remains dismal. The life expectancy of HCC patients is hard to predict because of the high possibility of postoperative recurrence. Many factors, such as patient's general conditions, macroscopic tumor morphology, as well as tumor hitopathology features (Qin and Tang, 2002). Co-existing hepatitis status and liver cirrhosis are other important factors influencing the prognosis of HCC patients. The inflammatory activity and hepatic reserve have been confirmed as risk factors for recurrence. Longer disease-free survival (DFS) is found in patients without active hepatitis, and suppression of co-existing hepatitis is necessary to achieve better DFS (Takata, 2000).

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Inflammatory cell infiltration

Marked inflammatory cell infiltration in the tumor could predict a better prognosis, which could attribute to the anti-tumor effect induced by cellular immunity of CD8+ and CD4+ T lymphocytes. Wada *et al*, found the patients with HCCs less than 3 cm in diameter with marked inflammatory cell infiltration had a much lower recurrence rate after resection and a higher 5-year survival rate compared with the controls (Wada *et al*., 1998). The incidence of grade 2/3 and higher HAI was higher in patients with viral hepatitis C (Nanashima *et al*., 2003). Treatment with RAGE blocking antibody

blocked the enhanced viability, invasion, clone formation and tumor growth *in vivo* resulted by S100A9, suggesting that these effects were mediated via RAGE ligation (Wu *et al*., 2015).

MATERIALS AND METHODS

We have studied biopsy material from 32 patient suffering of hepatocellular carcinoma and underwent resection of malignant and peri-malignant tissue. Sections from these biopsies were subjected to the following procedures:

1) Routine histopathological examination using paraffin sections stained by hematoxylin and eosin stain, with special reference to:

- Diagnosis of hepatitis grade (Hepatitis Activity Index HAI) and stage of fibrosis (Ishak *et al*., 1995).
- Diagnosis of malignancy; grade and pattern of hepatocellular carcinoma
- Diagnosis of hepatocellular dysplasia.

2) Immunohistochemical study of tissue sections using monoclonal antibody against RAGE protein.

(0.04% bovine serum albumin, A2153, Sigma-Aldrich, Shanghai, China, and 0.5% normal goat serum X0907, Dako Corporation, Carpinteria, CA, USA, in PBS) for 30 min at room temperature. Anti-*RAGE* antibody (A11): sc- 80652 RAGE Antibody (A11) is a mouse monoclonal IgG_{2a} provided at 200 µg/ml, raised against a truncated extracellular domain of RAGE of human origin (Santa Cruz Biotechnology, USA). The antibody was used at a dilution of 1:100. The antibody was incubated overnight at 4°C. Sections were then washed three times for 5 min in PBS. Non-specific staining was blocked 5% normal serum for 30 min at room temperature. Finally, staining was developed with diaminobenzidine substrate and sections were counterstained with hematoxylin. PBS replaced RAGE antibody in negative controls.

Quantification of protein expression

The expression of *RAGE* was semi quantitatively estimated as the total membrano-cytoplasmic immunostaining scores, which were calculated as the product of a proportion score and an intensity score. The proportion and intensity of staining was evaluated independently. The proportion score reflected the fraction of positive staining cells (score 0: <5%, score 1: 5%-10%, score 2: 10%-50%, score 3: 50%-75%, score 4: >75%),

Table 1. Distribution of studied cases in relation to scores of RAGE expression

			NRAGE score		Total
			Low	High	
Lesion	Peri-malignant	Count	28	4*	32
		%	87.5%	12.5%	100.0%
	Dysplasia	Count	4	16	20
		%	20.0%	80%	100.0%
	HCC	Count	8	24	32
		%	25.0%	75%	100.0%
Total		Count	40	44	84
		%	47.7	52.3%	100.0%

* Significant difference from dysplastic and HCC cases, p<0.01

Table 2. Differences of RAGE scores in relation to grades of HAI and in cirrhotic and non-cirrhotic peri-malignant liver tissue

			RAGE scores		Total
			Low Score	High Score	
Hepatitis Activity Index (HAI)	1	Count	4	0.0	4
		%	100%	0%	100%
	2	Count	4	4	8
		%	50%	50%	100%
	3	Count	8	12	20
		%	40.0%	60.0%	100%
Total		Count	16	16	32
		%	50%	50%	100%
Stage of Fibrosis	Cirrhosis (5-6)	Count	10	8	18
		%	55.56%	44.44%	100%
	Fibrosis (1-4)	Count	6	8	14
		%	42.86%	57.14%	100%
Total		Count	16	16	32
		%	50%	50%	100%

Non significant difference (p>0.05)

Immunohistochemical Method

Anti-RAGE antibody (Santa Cruz Biotechnology) was used for immunohistochemical (IHC) detection of the expression of *RAGE* protein in tissue. Tissue sections were processed for IHC analysis of *RAGE* protein as follows. IHC examinations were carried out on 3 µm thick sections. For anti-RAGE IHC, unmasking was performed with 10 mM sodium citrate buffer, pH 6.0, at 90°C for 30 min. Sections were incubated in 0.03% hydrogen peroxide for 10 min at room temperature, to remove endogenous peroxidase activity, and then in blocking serum

and the intensity score represented the staining intensity (score 0: no staining, score 1: weak positive, score 2: moderate positive, score 3: strong positive). Finally, a total expression score was given ranging from 0 to 12. Based on the analysis in advance, *RAGE* was regarded as negative expression in gastric cancer tissues if the score <2, and positive expression if the score ≥2. (Dai *et al*., 2014)

RESULTS

Our study includes biopsy material from 22 males and 10 females presented to the surgery department as cases of

hepatocllular carcinoma for partial hepatectomy, with the mean age 46.38 years for males and 54.37 years for females, showing significant difference ($p<0.01$). Peri-malignant liver tissue sections showed lower scores of RAGE expression compared to either dysplastic or hepatocellular carcinoma tissues, with significant difference ($p<0.01$) (Table 1). In peri-malignant tissue sections the score of RAGE expression increased with increasing grades of HAI. Peri-malignant tissue sections of non-cirrhotic liver parenchyma showed higher scores of RAGE expression compared to cirrhotic liver sections, with nonsignificant difference (Table 2).

Most malignant tissue specimens showed mild intra-tumorous inflammatory cellular infiltration. RAGE score increased with increased intensity of inflammation, however, this difference is non-significant ($p>0.05$) (Table 3). Most cases of liver cell dysplasia and hepatocellular carcinoma showed high scores of RAGE expression. The percentage of hepatocellular carcinoma cases with high scores of RAGE expression is higher in low and moderate grades of malignancy compared to higher grades, however, this difference is non-significant ($p>0.05$). (Table 4)

Table 3. Relation between intra-tumorous inflammatory infiltration and score of RAGE expression

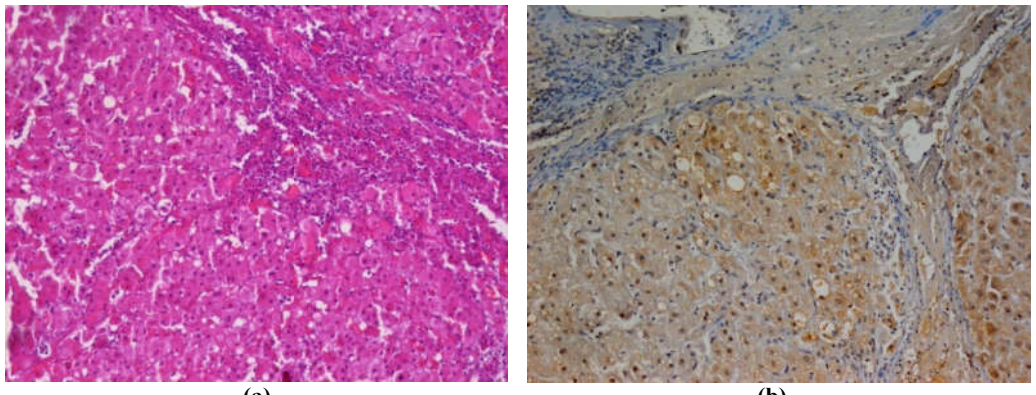
			RAGE score		Total
			Low	High	
Intra-tumorous inflammatory infiltration	Mild	Count	7_a	17_a	24
		%	29.17%	70.83%	100.0%
	Moderate	Count	1_a	4_a	5
		%	20.0%	80.0%	100.0%
	Marked	Count	0_a	3_a	3
		%	0.0%	100.0%	100.0%
Total		Count	8	24	32
		%	25.0%	75.0%	100.0%

Each subscript letter denotes a subset of RAGE score categories whose column proportions do not differ significantly from each other at the .05 level.

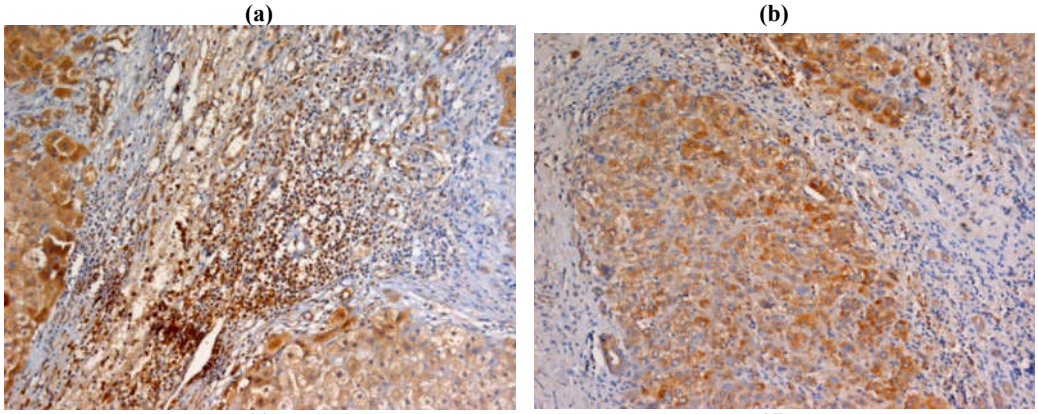
Table 4. Distribution of cases of dysplasia and different grades of hepatocellular carcinoma in relation to RAGE expression scores

			RAGE score		Total
			Low	High	
Tumour Grade	Dysplasia	Count	4	16	20
		%	20.0%	80%	100.0%
	1	Count	4	14	18
		%	22.22%	77.78%	100.0%
	2	Count	2	8	10
		%	20.0%	80.0%	100.0%
	3	Count	2	2	4
		%	50.0%	50.0%	100.0%
Total		Count	12	40	52
		%	23.1%	76.9%	100.0%

Non-Significant Difference within columns



(a) **(b)**



(c) **(d)**

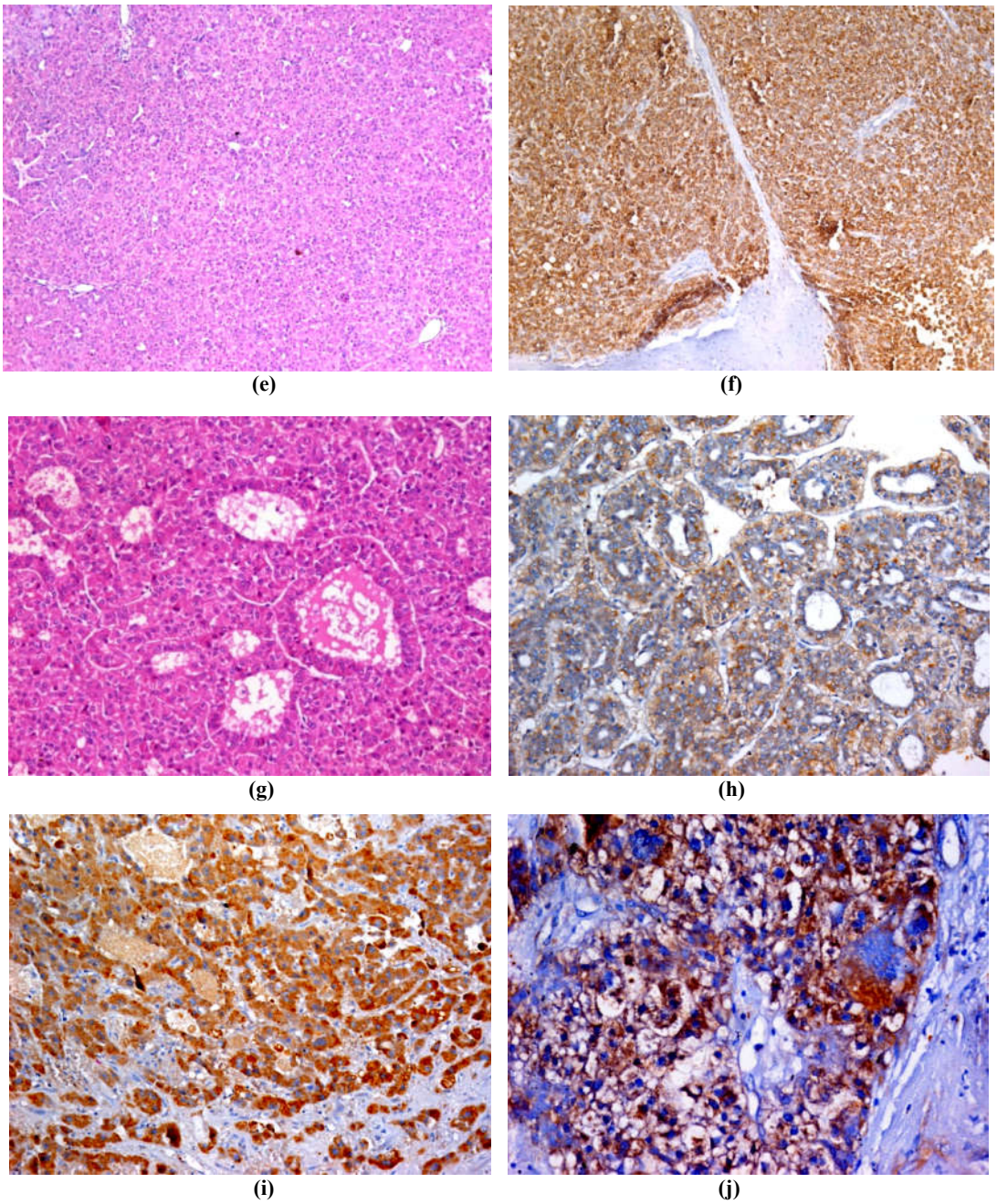


Fig. (a) Section in a case of HCV hepatitis of mild activity (H&E stainX100)
(b) Mild cytoplasmic expression of RAGE in case of HCV hepatitis with mild activity, with periportal distribution (IHC, RAGE-DAB,X100)
(c) Section in a case of HCV hepatitis of moderate activity, showing peri-portal moderate expression of RAGE (IHC, RAGE-DAB,X100)
(d) Heterogenous moderate and marked cytoplasmic expression of RAGE in case of HCV hepatitis with established cirrhosis (IHC, RAGE-DAB,X100)
(e) Section in a case of HCV hepatitis with small cell dysplasia in peri-malignant tissue (H&E stain, X100)
(f) Diffuse marked cytoplasmic expression of RAGE in case of HCV hepatitis with small cell dysplasia (IHC, RAGE-DAB,X100)
(g) Section in a case of low grade HCC with focal acinar pattern (H&E stain, X100).
(h) Mild cytoplasmic expression of RAGE in case of low grade HCC (IHC, RAGE-DAB,X100)
(i): Section in case of moderately differentiated HCC showing high score for RAGE expression. (IHC, RAGE-DAB, X200)
(j): Section in case of high grade HCC with clear cell pattern showing moderate focal cytoplasmic expression for RAGE. (IHC, RAGE-DAB, X200)

DISCUSSION

Hepatocellular carcinoma (HCC) is the most common primary hepatic malignancy of adults. It is the sixth most common cancer worldwide and the third most common cause of cancer death (Ding and Wang, 2014). In Egypt, liver cancer forms 11.75% of the malignancies of all digestive organs and 1.68% of the total malignancies. HCC constitutes 70.48% of all liver tumors among Egyptians (Lehman *et al*., 2008). It was reported that hepatocellular carcinoma (HCC) accounts for over 90% of all primary liver cancers.

With an ever increasing incidence trend year by year. Hepatic resection is generally considered to be one of the most effective therapies for HCC patients (Wu *et al*., 2015). Our study includes biopsy material from 24 males (75%) and 8 (25%) females presented to the surgery department as cases of hepatocellular carcinoma for partial hepatectomy. The mean age of our patients was 46.38 years for males and 54.37 years for females with significant difference between both sexes ($p<0.01$). Sex and age distribution of our cases was comparable to what was reported by (Holah *et al*., 2015) who revealed that 51.1% of their studied HCC patients were at least

58 years old, 81.5% male and 18.5% female. Darbari *et al.* (2003) reported that regardless of the geographic location, HCC occurs more frequently in men than in women, with the male : female ratios in various countries ranging from 2 : 1 to 5 : 1. The precise reason is not known, but it has been shown that many tumors have androgen receptors, and there is also a male predominance in risk factors. (Darbari, 2003) The receptor for advanced glycation end products (RAGE) is a multiligand receptor classified as an immunoglobulin superfamily cell-surface molecule. It is recognized to be responsible for cancer progression in several human cancers (Vlassara *et al*., 1989). In our work, peri-malignant liver tissue sections with chronic hepatitis C showed 7 negative cases (21.88%) and lower scores of RAGE expression compared to hepatocellular carcinoma tissues, with significant difference ($p<0.01$). Foci of dysplasia show high scores of RAGE expression, with significant difference compared to non-malignant non-dysplastic hepatitis lesions. On his study on liver tissue, Hiwatashi *et al*. (2008) found that the RAGE levels were different in different hepatic lesions: they were lower in the normal subjects versus the subjects with hepatitis and were highest in the subjects with HCC (Hiwatashi *et al*., 2007). In our study, 50% of peri-malignant tissue (hepatitis) were associated with high grades of HAI. The percentage was higher (60%) in foci of dysplasia. Most cases of it was previously reported by (El-Serag *et al*., 2007) that HCC are inflammation-related malignancies, which develop as a consequence of underlying liver disease, mostly viral hepatitis. (El-Serag, 2007).

In general, cancer-related inflammatory responses affect cellular proliferation, cell survival, angiogenesis, tumor cell migration, invasion, metastasis and inhibition of adaptive immunity, indicating that cancer and inflammation are closely associated (Sica *et al*., 2008). We found that in peri-malignant tissue sections the score of RAGE expression increased with increasing grades of HAI. Some literature reports the role of RAGE on various types of liver diseases, such as chronic hepatitis and liver cirrhosis in eliciting oxidative stress generation and a subsequent inflammatory response that prolong inflammation and apoptosis (Hyogo and Yamagishi, 2008; Basta *et al*., 2011). Hiwatashi *et al*. (2008) reported a significant difference in the levels of RAGE expression was observed between hepatitis virus-positive and virus-negative tissues ($p <0.01$) in the study of noncancerous inflammatory liver tissues (i.e., chronic hepatitis or liver cirrhosis) (Hiwatashi *et al*., 2007). Our results showed that in cases of hepatocellular carcinoma, most of the studied sections whether cirrhotic or non-cirrhotic showed high scores of RAGE expression. This finding was in accordance with the results of Ito *et al*. (2014) who showed that there was no significant relationship between RAGE positivity in tumor and background liver status, (Ito *et al*., 2014).

Our results showed also that most malignant tissue specimens showed mild intra-tumorous inflammatory cellular infiltration, however, RAGE score increased with increased intensity of inflammation. This was in accordance of the findings of Hiwatashi *et al*. (2008) who provided a new hypothetical concept that hepatic RAGE expression may be relevant to the stage or severity of inflammation and the incidence of carcinogenesis and early tumorigenesis of HCC (Hiwatashi *et al*., 2007). An underlying cause of HCC is continuous infection with the hepatitis virus, i.e., it is strongly associated with inflammation. Additionally, many studies have revealed that aberrant inflammatory molecules and inactivation of inflammatory pathways are major players in liver carcinogenesis (Ding and Wang, 2014; Szabo and Lippai, 2012). Therefore, the measurement of inflammatory markers should be clinically useful. One aim of the present study was to investigate the usefulness of RAGE expression as a prognostic factor for HCC. We found also that most cases of hepatocellular dysplasia and hepatocellular carcinoma showed high scores of RAGE expression. The percentage of hepatocellular carcinoma cases with high scores of RAGE expression is higher in low and moderate grades of malignancy compared to higher grades. Hiwatashi *et al*. (2008) reported that RAGE messenger RNA expression was high in well differentiated and moderately differentiated tumors but declined in poorly differentiated HCC.

The study concluded that early stage HCC with decreased blood supply may acquire resistance to stringent hypoxic milieu through hypoxia induced RAGE expression. (Hiwatashi *et al*., 2007) We concluded that HCV induced HCC showed higher immunohistochemical expression of RAGE compared to peri-malignant tissue. RAGE expression correlated with grade of hepatitis and with the intensity of intra-tumorous inflammatory cellular infiltration. Cellular expression of RAGE was higher in dysplasia as well as low and moderately differentiated HCC compared to poorly differentiated ones. It was suggested that the blockade of RAGE may limit harmful inflammatory mechanisms and thereby facilitate repair in the injured liver (Basta *et al*., 2011). It was also suggested that RAGE in HCC may be a novel, independent biochemical marker with the capacity to predict therapeutic outcome and may also be a new therapeutic target for patients with HCC after hepatectomy. (Ito *et al*., 2014) Our findings supported these previous suggestions. We recommended that these findings should be confirmed and validated in future studies.

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