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Original Article

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Immunohistochemical Study of Some Biological Markers Which Can Be Targeted by New Anticancer Therapies in Hepatocellular Carcinoma

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ABSTRACT

Aim: The aim is to study VEGF, HER2/neu, EGFR and c-kit expression by immunohistochemistry in HCC in Egyptian cirrhotic patients.

Patients & Methods: Fifty four cases of HCC were included in this study, 38 cases of them were hepatectomy samples. All were histologically diagnosed and graded. There were 10 cases well differentiated, 22 moderately differentiated, 16 poorly differentiated and 6 undifferentiated HCC.

Results: All patients had cirrhotic livers, and the most common etiology was HCV (59%). Immunohistochemical expression of VEGF, HER2/neu, c-kit and EGFR in HCC cases were studied with scoring of each case. Statistical analysis was done to correlate the results. There were 48/54 (88.9%) VEGF, 22/54 (40.7%) HER2/neu, EGFR 6/54 (11%) and 28/54 (70.4%) c-kit positive cases. There was statistical significant correlation between expression of VEGF and histopathological grading of HCC but, it was statistically insignificant with the others

Conclusion: VEGF is an important molecular target for anti angiogenic therapy in HCC patients.

Key words: vascular endothelial growth factor (VEGF), HER2/neu, c-kit, epidermal growth factor receptor (EGFR), hepatocellular carcinoma (HCC)

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INTRODUCTION

Targeted cancer therapies are drugs that block the growth and spread of cancer by interfering with specific molecules involved in tumor growth and progression which, may be more effective than other types of treatment, including chemotherapy and radiotherapy, and less harmful to normal cells.(1)

Many targeted cancer therapies have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of specific types of cancer as cancer breast and colon. Others are being studied in clinical trials , and many more are in preclinical testing. Targeted cancer therapies are being studied for use alone, in combination with other targeted therapies, and in combination with other cancer treatments, such as chemotherapy.(2)

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Liver cancer is the fifth most common cancer among men worldwide and eighth in women. It accounts for almost 4% of all human cancers. The highest incidence rates are recorded in China (55% of the total world), Japan, South East Asia and some regions in Africa including Egypt and sub-Saharan Africa [3].

According to Gharbiah population-based cancer registry published in 2007, the population of Egypt has a heavy burden of liver cancer. This is mainly due to increasing prevalence of hepatitis C viral infection. The hepatocellular carcinoma (HCC) represents 8.1% of all incident cancer in Egypt. It is responsible for 12.8% of male cancers, and 3.2% of female cancers, so ranked second in males after bladder carcinoma and seventh in females. The number of incident cases in Egypt is approximately 9360 patients every year. This places Egypt in the 90th percentile rank worldwide, which means that only 10% of all registries worldwide are worse than Egypt [4].

The prognosis of hepatocellular carcinoma will soon include molecular and genomic "fingerprints" that are unique to each cancer, which will allow more personalized treatment plans for patients as more targeted therapies become available.[5] So, the aim of this study is to evaluate the expression of some biological markers (VEGF, HER2/neu, EGFR and c-kit) of hepatocellular carcinoma using immuno-histochemistry as their expression may modify treatment regimens.

PATIENTS & METHODS

Fifty four HCC cases(38 hepatectomy specimens and 16 core biopsies) were collected during the period of 2009 and 2010 at Tropical Medicine Department, Faculty of medicine, Tanta University, Egypt.

- Cases with hepatic focal lesion on ultrasonography with elevated alpha-fetoprotein (AFP) and confirmed to be HCC with triphasic CT scan were sent to surgery for hepatectomy (38 cases).
- Sixteen cases of core biopsy for patients with inclusive imaging results and normal alpha-fetoprotein.

The risk factors as cirrhosis, HCV, HBV either individually or co-infection of all the studied cases were studied and evaluated.

All the studied specimens were fixed in 10% formalin and subsequently embedded in paraffin, then submitted to haematoxylin and eosin (H&E) staining for assessment of the pathological parameters of HCC including the tumour grade, steatosis, as well as the histopathological pattern and cytological features. The adjacent non tumorous liver tissue was also examined for the presence of cirrhosis.

Grading of HCC cases was done according to the World Health Organization (WHO) into well, moderately, poorly and undifferentiated tumours[6].

Immunohistochemical staining was performed on 3–5 µm sections using the Ultra Vision One Detection System (HRP Polymer&DAB Plus Chromogen, Catalog #TL-015-HDJ, LabVision, USA). Sections were deparaffinized with xylene and rehydrated with graded alcohol series. Antigen retrieval was done by immersing the sections in 10mmol/l citrate buffer (pH6.0) for 10 minutes at 100 1C° in microwave. Endogenous peroxidase activity was blocked with hydrogen peroxide block for 10 minutes. After thorough washing of the sections with phosphate buffered saline, incubation was done for 10 minutes with UltraV block to prevent non-specific background staining, followed by rinsing the sections with PBS. Subsequently, an overnight incubation of the sections with the following antibodies was done at room temperature in a humidity chamber: [1] Mouse monoclonal antibody against vascular endothelial growth factor against "VEGF" (Ab-7 "Clone VG1", Catalog # MS-1467-P, Lab Vision, USA) [2] Mouse monoclonal antibody against epidermal growth factor receptor "EGFR" (Ab-10 "Clone 111.6", Catalog # MS-378-B, Lab Vision, USA) [3] Mouse monoclonal antibody against Her-2/neu (Clone e2-4001+3B5, Catalog # MS-

730-P, LabVision, USA) and [5] Rabbit polyclonal antibody against CD117 "c-kit" (Ab-6, Catalog # RB-1518-P, Lab Vision, USA).

The sections were then washed with PBS and incubated with biotinylated goat anti-polyvalent (secondary antibody) for 10 minutes at room temperature followed by washing with PBS, then incubated with streptavidin peroxidase solution for 10 minutes at room temperature, then rinsed with PBS. The reaction products were visualized using 3-30-diamino-benzidine-tetra-hydrochloride (DAB), and the sections were then counterstained with Mayer's haematoxylin, dehydrated in alcohol and mounted in Di-n-butyl-phthalate-polystyrene-xylene (DPX).

Positive controls: In order to confirm the results of the staining, positive controls were performed as follows: sections from a case of angiosarcoma "for VEGF", sections from a case of squamous cell carcinoma "for EGFR", sections from a case of Her-2/neu positive breast carcinoma "for Her-2/neu", and sections from a case of gastrointestinal stromal tumor "GIST" for CD117 "c-Kit". In addition,

Negative controls: omission of the primary antibodies was done, and instead, normal rabbit serum was applied.

Scoring of VEGF immuno-reactivity: We applied for all cases VEGF scoring system described and used by Raica et al. follows: negative staining with 0, weak positive (+1, weak reaction in less than 10% of tumor cells, moderate positive (+2, weak-moderate reaction in 10–50% of tumor cells) and intense positive (+3, strong or moderate intensity in more than 50% of tumor cells).[7]

Scoring of Her-2/neu immuno-reactivity: As nearly all cases expressed cytoplasmic HER2/neu scoring of cytoplasmic expression of HER2/neu was done. Cytoplasmic staining was evaluated in the neoplastic cells and quantified and classified it in four categories, such as more than 50%, 10-50%, less than 10% and negative.[8]

Scoring of EGFR immuno-reactivity: Membrane staining was evaluated in the neoplastic cells and quantified and graded as recommended in the detection kit: 0 score (Negative): No staining observed, or membrane staining in <10% neoplastic cells.

1+ score: Weak complete and/or incomplete membrane staining in >10% neoplastic cells. 2+ score: Moderate complete and/or incomplete membrane staining in >10% neoplastic cells. 3+ score: Strong complete and/or incomplete membrane staining in >10% neoplastic cells. Positive Scores (1+, 2+, and 3+ were considered positive)[9]

Scoring of c-kit immuno-reactivity: For c-kit expression level: score 0, no staining was observed or staining was observed in less than 10% of cells; score 1+, the cytoplasm was discretely and weakly-moderately stained in 10% or more of the epithelial cells; score 2+, the cytoplasm was strongly stained with or without membrane staining in 10% or more of the epithelial cells. Cases with a score of 1+ and 2+ were considered positive [10].

Statistical analysis: The collected data were statistically analyzed using SPSS statistical software package (SPSS 16.0 for Windows, SPSS Inc., Chicago, IL, USA). Chi square and Fisher exact probability tests have been used to compare between groups. A significant difference was considered when p value was <0.05.

RESULTS

Clinical results: In the studied group, median age was 62 years (40 to 88) and the male/female ratio was 40/14. Evaluation of risk factors showed that, all patients had cirrhotic livers, and the most common etiology was HCV (59%) as shown in table (1).

Histopathological results: Histopathological examination of the tissue sections of 54 cases showed that there were 10 cases of well differentiated (HCC G1), 22 cases of moderately differentiated (HCC G2), 16 cases of poorly

differentiated (HCC G2) and 6 cases of undifferentiated (HCC G4).

The histological patterns were pure trabecular, pseudoacinar and mixed. Macrovesicular steatosis was detected in 6 cases. Adjacent cirrhosis was detected in all cases.

Immunohistochemical results (Table 2)

• **VEGF:** there were 48 out of 54 cases positive (88.9%) for anti-VEGF antibodies and there were 6 (11.1%) cases negative for VEGF. The positive cases showed cytoplasmic staining variable in intensity and percentage (fig 1, 2). 22/48 positive cases (45.8 %) were of score 3, 14/48 cases (29.2 %) of score 2 and 12/48 cases (25%) of score 1.

• **HER2/neu** there were 32/54 (59.3%) cases negative for immunostaining of anti- HER2/neu antibodies, while 22/54(40.7%) cases were positive for HER2/neu. Positive cases showed granular cytoplasmic staining with score ranged from (1-3). 18/22 cases (81.8%) showed positivity of score3 (fig 3). 2/22 cases (9.1%) for both score 2 and of score 1. Six cases showed incomplete membranous staining beside cytoplasmic staining (fig 4).

• **EGFR:** there were 6/54(11%) cases positive for anti-EGFR antibodies and there were 48/54(89%) cases negative for EGFR. The positive cases showed weak incomplete membrane staining in >10% neoplastic cells i.e score1 (fig 5)

• **C-kit:** there were 38/54 (70.4%) cases positive for anti c-kit antibodies and 16/54 (29.6%) cases negative. The positive cases showed cytoplasmic positivity (fig 6) and 12 cases showed membranous staining (fig 7). There were 24/38 positive cases (63.2%) of score 2 and 14 positive cases (36.8%) of score 1.

Relationship between VEGF expression and grading of HCC (table 3)**:** Expression score (3&2) of VEGF was high in well differentiated HCC (90%), and decreased with increasing of grade HCC. It was 63.7% with moderately differentiated HCC and 33.3 % with poorly differentiated HCC.

There was statistical significant correlation between expression of VEGF and histopathological grading of HCC, p value was <0.05. No statistical significant correlation has been found between (HER2/neu, EGFR and c-kit) expression and histopathological grading of HC

Table (1): Risk factors of HCC in studied group (54 cases)

Risk factors	Patients	(54 cases)
HCV	31	59%
HBV	15	26%
HCV & HBV co-infection	8	15%
Cirrhosis	54	100%

Table (2): Summary of immunohistochemical results (54 cases)

	Total positive cases (54cases)	Scoring of positive cases			Total negative cases (54 cases)
		Score1	Score2	Score3	
VEGF	**48** Cases (88.9%)	12 (25%)	14 (29.2%)	22 (45.8 %)	**7** (11.1%)
HER2/neu	**22** Cases (40.7%)	2 (9.1%)	2 (9.1%)	18 (81.8%)	**32** (59.3%)
EGFR	**6** Cases (11%)	6 (100%)	0	0	**48**(89%)
c-Kit	**38** Cases (70.4%)	14 (36.8)	24 (63.2%)	0	**16** (29.6%)

Table (3): Relationship between VEGF expression and histopathological grading (54 cases)

	VEGF					P*
	Negative	Weak (score 1)	Moderate (score 2)	Strong (score 3)	Total	
Well Differentiated	**0**	**1**	**3**	**6**	**10**	0.01
	.0%	10%	30.0%	60.0%	100.0%	
Moderately Differentiated	**1**	**7**	**6**	**8**	**22**	
	4.5%	31.8%	27.3%	36.4%	100.0%	
Poorly Differentiated	**1**	**4**	**3**	**8**	**16**	
	6.2%	25%	18.8%	50%	100.0%	
Undifferentiated	**4**	**0**	**2**	**0**	**6**	
	66.7%	.0%	33.3%	0%	100.0%	
Total	**6**	**12**	**14**	**22**	**54**	
	11.1%	22.2%	25.9%	40.7%	100.0%	

*significant using Pearson Chi-Square 2 sided test

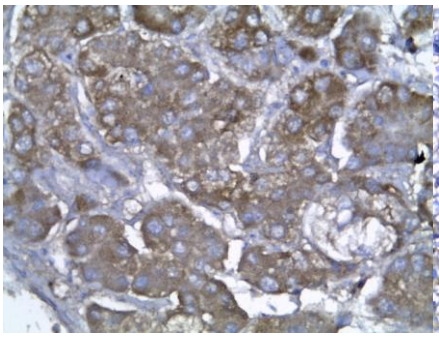

Fig (1): VEGF cytoplasmic strong expression score 3 in moderately differentiated HCC (original magnification x40)

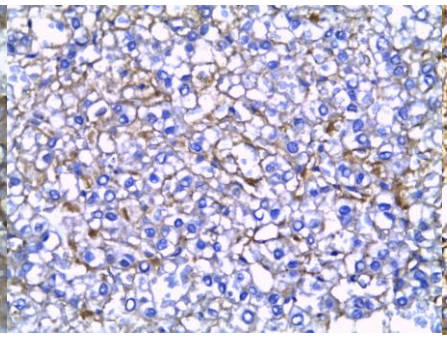

Fig (2): VEGF expression in clear type HCC (original magnificationx40)

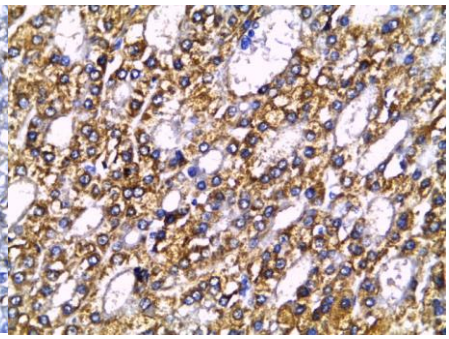

Fig (3): HER2/neu expression of score 3 with granular cytoplasmic staining in HCC grade2 (original magnification x40)

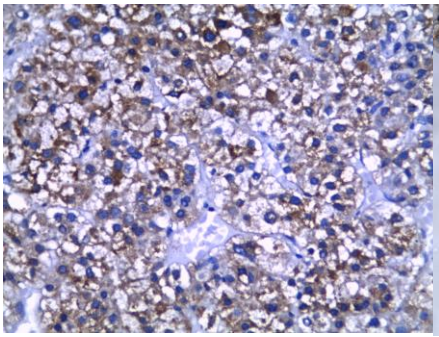

Fig (4): HER2/neu expression in HCC grade 2 showing granular cytoplasmic and incomplete membranous staining (original magnificationx40)

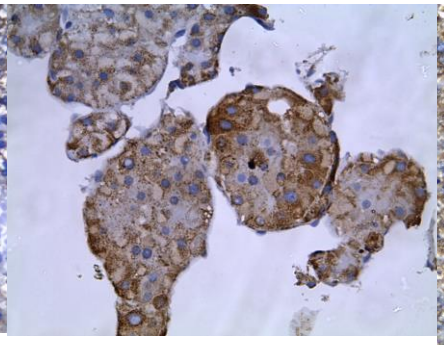

Fig (5): EGFR expression in HCC with weak incomplete membranous staining of score 1(original magnificationx40)

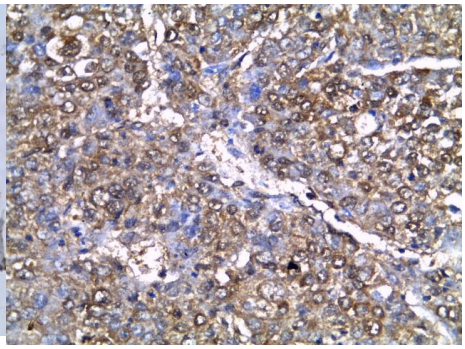

Fig (6): c-kit cytoplasmic expression in HCC (original magnificationx40)

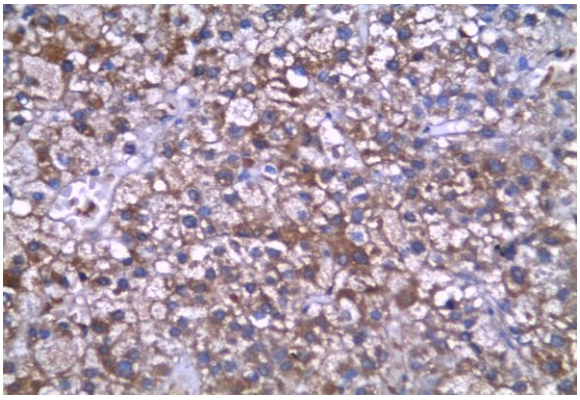

Fig (7): c-kit cytoplasmic and membranous staining in HCC (original magnificationx40)

DISCUSSION

Hepatocellular carcinoma is the sixth most prevalent cancer and the third most frequent cause of cancer-related death. Infection with hepatitis B Virus (HBV) or hepatitis C Virus (HCV) in human liver induces the development of chronic hepatitis, liver cirrhosis, and in some instances hepatocellular carcinoma (HCC). HCV may promote cancer through cirrhosis, which is often associated with HCV-related HCC, but it might also have oncogenic properties by interacting with cellular genes that regulate cell growth and differentiation.[11]

HCC, with and without HCV infection, represents a major health problem in Egypt, where the two pathological conditions are integrated in many cases. Many reports suggested that HCV infection is, directly or indirectly, involved in HCV-mediated HCC.[12]

In the present study, all HCC patients had cirrhotic livers, and the most common etiology was HCV (59%).

The rate of hepatocellular carcinoma (HCC) is increasing in Egypt where the major risk factors are chronic infections with hepatitis B and C viruses (HBV and HCV). Infection with HCV and HCV-HBV double infection, but not HBV or HEV infection alone, is strongly correlated with HCC in Egypt.[13]

Also, El-Zayadi et al stated that, there was nearly a twofold increase of the proportion of HCC among chronic liver disease patients in Egypt with a significant decline of HBV and slight increase of HCV as risk factors and that cirrhosis is the common pathway by which several risk factors exert their carcinogenic effect [14]

Patients with cirrhosis are at highest risk of developing this malignant disease, and ultrasonography every 6 months is recommended. Surveillance with ultrasonography allows diagnosis at early stages when the tumour might be curable by resection.[15]

HCC is potentially curable by surgical resection and liver transplantation. However, the majority of patients present with advanced stage disease, which is most commonly accompanied by severe background liver disease. Therefore, surgery is feasible for only a small fraction of patients with localized disease. In the last decade, molecular characterization of HCC has led to the recognition of defined aberrant signaling pathways, which helped in subsequent development of targeted agents as potential choices for the treatment of this chemoresistant disease.[16]

In the present study VEGF was positive in about 89% of the studied cases which has statistical significant correlation with histopathological grading of HCC denoting that the targeted cancer therapies primarily targeting angiogenesis can be entered as a clinical trial to improve the disease outcomes.

Elgendy et al.[12] investigated 77 of HCC patients admitted to the National Cancer Institute, Cairo during the period 2002-2003. HCC patients were divided into in HCV related HCC patients and HCV-free HCC patients. The plasma circulating levels VEGF was dramatically elevated in all HCC patients. Also they noticed that, in presence of HCV infection, the elevation of VEGF was significant compared to healthy subjects but insignificant compared to the corresponding HCV-free patients

Gershtei etal[17] Compared the level of VEGF in tumerous tissue of different grades HCC compared to adjacent liver tissue by immune-histopathological method and by enzyme immunoassay. They found that, expression of VEGF was Increased in HCC tissue compared to adjacent liver tissue which was correlated with the degree of histological differentiation and stage of the tumor.

Also, Mathonnet et al [18] they found high expression of VEGF in HCC. They suggested a possible role of angiogenesis in HCC carcinogenesis. On the contrary Heo etal[19] stated that there was no correlation between differentiation of HCC and the VEGF expression

It is well known that HCC is a vascular tumor and is dependent on angiogenesis for growth. Important growth factors include vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), angiopoietins and fibroblast growth factors.[20]

Disruption of the VEGFR pathway and targeting growth factors that drive the angiogenic process can thus interrupt effective angiogenesis and have clinical effect in the treatment of HCC. Anti-angiogenic drugs such as sorafenib and bevacizumab target different points along the VEGFR pathway[20].

Bevacizumab (Avastin; Genentech, Inc, South San Francisco, CA, USA) is a recombinant humanized monoclonal antibody directed against VEGF. Bevacizumab is also used in the treatment of other malignancies including colon, breast and kidney cancer. It has been studied both as a single agent, as well as in combination with chemotherapeutic drugs.[20] HER-2/neu expression has become an important biomarker for identifying patients who could respond to HER-2/neu targeting therapy using the fully humanized monoclonal antibody trastuzumab. [21]

In the present study HER-2/ neu was considerably expressed being positive in (40.7%) of studied cases, showing cytoplasmic staining beside of the membranous staining, with score ranged from (1-3). 81.8% of positive cases showed positivity of score3. There was no significant correlation found between HER-2/neu expression and histopathological grading of HCC.

The relationship between HER-2/neu overexpression and gene amplification is well evaluated in breast cancers but remains unclear or controversial in many other tumor entities. Trastuzumab therapy is highly efficient in HER-2/neu amplified breast cancer both in metastatic disease and as an adjuvant therapy [21]. Tapia et al[22] tested the HER-2/neu status in more than 120 different tumor entities. They found strong association between HER-2/neu overexpression and amplification was seen many examined cancer entities. A variety of other tumor entities including HCC with often limited therapeutic options have similar patterns of HER-2/neu alterations as observed in breast cancer (ie high overexpression due to high level gene amplification). Such tumor entities should be carefully evaluated for a possible utility of trastuzumab treatment.

Immunohistochemical analysis revealed HER-2 over-expression as high as 92.3% in HCC by Tang et al[23] but no obvious relationship was observed between HER-2 expression and recurrence or migration of HCC .

On the contrary, eight hundred and sixty eight surgical samples from patients with primary HCC were examined for their HER-2/neu status with the immunohistochemical method and FISH analysis. There is a low frequency of HER-2/neu over expression/amplification in HCC. They suggested that, no role for HER-2/neu as a prognostic marker and no benefit of anti-HER-2/neu trastuzumab treatment in patients with HCC Xian et al[24].Also, Bacaksiz et al[25] stated that, although HER-2/Neu amplification is not the primary mechanism in the development of liver tumors, it might play a role in one of the steps of multistage carcinogenesis of HCC.

Cytoplasmic expression of HER-2/neu was reported in other cancer types as in colonic carcinomas. In their study Ghaffarzadegan et al[26] reported that HER2/neu was expressed in the cytoplasm of 65.9% of their colonic carcinomas cases, while 34.1% showed membranous expression and no single case showed pure cytoplasmic expression.

Different patterns of HER-2/neu staining were detected in gastroeosophageal carcinomas in the study of Boers et al[27]. Also, HER-2/neu expression in colonic adenocarcinomas, Twenty-seven (65.9%) cases had cytoplasmic and 14 (34.1%) cases had membranous (predominant) and cytoplasmic staining. There was no case with pure membranous staining [26].

In the study of Lee et al [28] on gastric carcinomas it was found that strong membrane staining for HER-2/neu was noted in 14 cases (25%), all of which were of the intestinal type. Only cytoplasmic staining was found in an additional 21 cases (37.5%).

In the present study, the least to be expressed was the EGFR in 11% only and by weak expression. There was no significant correlation found between histopathological grading and expression of EGFR. This may give an idea that the evaluation of EGFR has a less valuable role in targeted cancer therapy of HCC.

The epidermal growth factor receptor (EGFR) signaling pathway is an important mediator of cancer cell oncogenesis, proliferation, maintenance, and survival. For this reason, it has long been an attractive candidate as anticancer drug target, but showed only a minor effect in HCC [29]

In the study of Thomas et al [30] EGFR level in HCC was significantly lower than that of noncancerous liver tissues. They noticed that there was no obvious correlation between EGFR levels and other pathologic characteristics, such as tumor diameter, grade, extracapsular invasion, and vascular invasion .They concluded that EGFR is not a relevant oncogenic factor for HCC.

Although, Zhao et al [31] stated that the expression of EGFR mRNA was 60% in the tumour tissue of HCC but, without correlation with the diameter of tumor, the level of serum alpha-fetoprotein (AFP), the differentiation of tumor and the liver cirrhosis in the adjacent tissue.

Also, Buckley et al[32] found that EGFR was expressed in 40-70% of HCC and stated that, EGFR has relation to the initiation , progression and the recurrence of HCC. So, EGFR can be considered as a marker for predicting the metastasis and recurrence of HCC.

In the present study c-kit was expressed in 70% of the cases. All the positive cases were of score (2&1). There was no significant correlation found between of c-kit expression and histopathological grading of HCC.

This findings was in accordance with study of Mansuroqlu et al[33] C-kit expression was detected immunohistochemically in 70% of HCC with different degrees of intensity. Moreover, c-kit expression could also be found in about 90% of the corresponding peritumoral non cirrhotic as well as in cirrhotic liver tissues. C-kit mRNA was detectable in 83% of HCC and in 75% of the corresponding peritumoral noncirrhotic as well as in 100% of corresponding peritumoral cirrhotic samples. On the other hand Becker et al [34] stated that the overall percentage of positive immunohistochemical staining of HCC for c-kit expression was 2.3%.

c-kit was frequently evaluated on larger scale because targeted therapy using imatinib mesylate is now widely and successfully used in treatment of gastrointestinal stromal tumours (GIST).[35]

CONCLUSIONS

This study showed that, VEGF was over expressed in HCC studied cases followed by (HER-2/neu, c-kit) and EGFR which showed low expression.

- Current data suggested that, (VEGF) plays a critical role in angiogenesis of HCC. VEGF is an important molecular target for anti angiogenic therapy.
- (HER-2/neu and c-kit should be carefully evaluated on large scale for a possible utility of trastuzumab and Imatinib mesylate treatment respectively in HCC patients.
- There would be no benefit of anti EGFR treatments in patients with HCC.

Competing Interests: None

REFERENCES

1.Beutler J. Targeted Cancer Therapies Center for Cancer Research, National Cancer Institute. Feb 24, 2012 15:30.

2.Vitale A., Navaglia F., Morales R, Frigo A., Basso D., D'Amico D., and Zanus G. Molecular Refinement of Clinical Staging in Hepatocellular Carcinoma Patients Evaluated for Potentially Curative Therapies .Pub Med central PLoS One.2011; 6 (9).

3.Ferlay J., Bray F., Parkin D., and Pisani P. Cancer incidence and mortality worldwide In: Globocan 2000: IARC Cancer Bases No.5, editors. Lyon, IARC press 2001.

4.El-Hattab O. Cancer database, Egypt 1985-2001. In: World Health Organization. El-Hattab-O,editor. 2002.

5.D'Avola D., Iñarrairaegui M., Pardo F., Rotellar F., Marti P., Bilbao J., Martinez-Cuesta A., Benito A.,

Alegre F., and Mauleón E. Prognosis of hepatocellular carcinoma in relation to treatment across BCLC stages. Ann Surg Oncol. 2011 Jul; 18(7):1964-71.

6.Hamilton S. and Aaltonen L. Pathology and Genetics of Tumors of the digestive System. In: World Health Organization classification of tumors. Hamilton SR and Aaltonen LA, editors. JARC Press: Lyon 2000.

7.Raica M., Cimpean A., and Anghel A. Immunohistochemical expression of vascular endothelial growth factor (VEGF) does not correlate with microvessel density in renal cell carcinoma. Neoplasm 2007; 54(4):278–84.

8.Horiguchi S., Hishima T., Hayashi Y., Shiozawa Y., Horiguchi K., Kuroi K., et al. HER-2/neu cytoplasmic staining is correlated with neuroendocrine differentiation in breast carcinoma. J. Med Dent Sci 2010；57：155－63.

9.Gamboa-Dominguez A., Dominguez-Fonseca C., Quintanilla-Martinez L., Reyes-Gutierrez E., Green D, and Angeles-Angeles A., Epidermal growth factor receptor expression correlates with poor survival in gastric adenocarcinoma from Mexican patients: a multivariate analysis using a standardized immunohistochemical detection system. Modern Pathology 2004; 17: 579–87

10. Kondi-Pafiti A., Arkadopoulos N., Gennatas C., Michalaki V., Frangou-Plegmenou M., and Chatzipantelis P. Expression of c-kit in common benign and malignant breast lesions. Tumori 2010; 96: 978-84.

11. Tanaka Y, Kurbanov F., Mano S., et al. Molecular tracing of the global hepatitis C virus epidemic predicts regional patterns of hepatocellular carcinomamortality. Gastroenterology. 2006;130:703-714.

12. Elgendy S., Hessien M., Elsherbiny M., El-Salam I., El-Attar I., and El-Asser A. :A Panel of Molecular Markers in Hepatitis C Virus-Related Hepatocellular Carcinoma Journal of the Egyptian Nat. Cancer Inst., Vol. 17, No. 4, December: 270-278, 2005.

13. Strickland G., Elhefni H., Salman T., Waked I., Abdel-Hamid M., Mikhail N., et al. Role of hepatitis C infection in chronic liver disease in Egypt. Am J Trop Med Hyg. 2007, 67 (4): 436-442.

14. El-Zayadi A., Badran H., Barakat E., Attia M., Shawky S., Mohamed M., Selim O., and Saeid A. Hepatocellular carcinoma in Egypt: A single center study over a decade. World J Gastroenterol 2005; 11(33): 5193-5198

15. Forner A., Reig M., and Bruix J. Current strategy for staging and treatment: the BCLC update and future prospects. Semin Liver Dis. 2010 Feb; 30(1):61-74.

16. Kaseb A, Hanbali A., Cotant M., Hassan M., Wollner I., and Philip P. Vascular endothelial growth factor in the management of hepatocellular carcinoma: a review of literature. Cancer. 2009 Nov 1;115(21):4895-906.

17. Gershtein E., Dubova E., Shchegolev A. and Kushkinskii N. Vascular Endothelial Growth Factor and Its Type 2 Receptor in Hepatocellular CarcinomaBulletin of Experimental Biology and Medicine Vol. 149, No. 6, pp. 689-692, June, 2010.

18. Mathonnet M., Descottes B., Valleix D., Labrousse F., and Denizot Y. VEGF in hepatocellular carcinoma and surrounding cirrhotic liver tissues. World J Gastroenterol 2006 February 7; 12(5): 830-31.

19. Heo S., Jeong Y, Shin s., Kim j., Lim H., Lee J., and Kang H., Apparent Diffusion Coefficient Value of Diffusion-Weighted Imaging for Hepatocellular Carcinoma: Correlation with the Histologic Differentiation and the Expression of Vascular Endothelial Growth Factor Korean J Radiol. 2010 May-Jun; 11(3): 295–303

20. Chua C. and Choo S. Targeted therapy in hepatocellular carcinoma. International Journal of Hepatology 2011; volume 2011, Article ID 348297, 11 pages.

21. Cuello M., Ettenberg S., Clark A., Keane M., Posner R., and Nau M. Down-regulation of the erbB-2 receptor by trastuzumab (herceptin) enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in breast and ovarian cancer cell lines that overexpress cerbB-2. Cancer Res 2010: 61:4892-900.

22. Tapia C., Glatz K., Novotny H., Lugli A., Horcic M., and Sauter G. Close association between HER-2 amplification and overexpression in human tumors of non-breast origin Modern Pathology (2007) 20, 192–198.

23. Tang Z. Treatment of hepatocellular carcinoma. Digestion 1998; 59:556–62.

24. Xian Z., Zhang S., Cong W., Wu W., and Wu M. Overexpression/ amplification of HER-2/neu is uncommon in hepatocellular carcinoma. J Clin Pathol. 2005 May; 58(5):500-3.

25. Bacaksiz A., Sahin F., Bilezikci B., and Yilmaz Z. Determination of HER-2/Neu Status in Hepatocellular

Carcinoma Cases Genetic Testing. June 2008, 12(2): 211-214. doi:10.1089/gte.2007.0095.

26. Ghaffarzadegan K., Sharifi N., Vosooghynia H., Shakeri T., Ghiasi T., Moghadam S., Kafi G., Lari S., and Nassiri G. HER2/neu expression in colon adenocarcinoma and its correlation with clinicopathologic variable. IJBMS Spring 2006 ; 9(1):64-9.

27. Boers J., Meeuwissen H., and Methorst N. HER/2 status in gastroeosophageal adenocarcinomas assessed by two rabbit monoclonal antibodies (SP3 and 4B5) and two in situ hybridization methods FISH and SISH. Histopathology Feb 2011; 58:383-94.

28. Lee E., Cibull M., Strodel W., and Haley J. Expression of HER-2/neu oncoprotein and epidermal growth factor receptor and prognosis in gastric carcinoma. Arch Pathol Lab Med 1994 Mar; 118(3):235-9.

29. Kim H. and Lim H. Novel EGFR-TK Inhibitor EKB-569 Inhibits Hepatocellular Carcinoma Cell Proliferation by AKT and MAPK Pathways J Korean Med Sci. 2011 Dec; 26(12):1563-1568.

30. Thomas M., Chadha R., Glover K., Wang X., Morris J., Brown T., Rashid A., Dancey J., and Abbruzzese L. Phase 2 study of erlotinib in patients with unresectable hepatocellular carcinoma. Cancer 2007;110:1059–1067.

31. Zhao Y., Cao J., Wu F., Ou C., Yuan W., Mo Q., Wei W., Li Y., and Liang A. Expression and significance of EGF mRNA and EGFR mRNA in hepatocellular carcinoma. 2004 Jul; 23(7):762-6

32. Buckley A., Burgart L., Sahai V., and Kakar S. Epidermal growth factor receptor expression and gene copy number in conventional hepatocellular carcinoma. Am J Clin Pathol 2008; 129:245-51.

33. Mansuroqlu T., Baumhoer D., Dudas J, Haller F., Cameron S., Lorf T., et al. Expression of stem cell factor c-kit in non tumoral and tumoral hepatic cells. Eur J Gastroenterol Hepatol 2009 Oct; 21(10):1206-11.

34. Becker G., Schmitt-Graeff A., Ertelt V, Blum H. , and Allgaier H. CD117 (c-kit) Expression in Human Hepatocellular Carcinoma. Clinical oncology 2007 **April; 19(3):204-8.**

35. Kondi-Pafiti A., Arkadopoulos N., Gennatas C., Michalaki V., Frangou-Plegmenou M., and Chatzipantelis P. Expression of c-kit in common benign and malignant breast lesions. Tumori 2010; 96: 978-84.