Evaluation of Immunohistochemical Staining for Hepatocyte Growth Factor and c-*Met* in Endometrial Adenocarcinoma

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ABSTRACT:

BACKGROUND:

Endometrial carcinoma is a primary malignant epithelial tumor, usually with glandular differentiation, arising in the endometrium with the potential to invade and metastasize. Hepatocyte growth factor (HGF) and its receptor c- *M*et have been implicated in uterine development, pregnancy, and endometrial disorders, such as endometriosis and carcinoma.

OBJECTIVE:

The goal of this study was to evaluate immunohistochemical (IHC) staining patterns of HGF and c-*Met* in endometrial adenocarcinoma and to correlate staining with the biological behavior and outcome of endometrial adenocarcinoma.

PATIENTS AND METHOD:

A retrospective study included 45 cases ,who underwent total abdominal hysterectomy and bilateral salpingo-oopherectomy, between 2005-2010 samples ,were taken from Teaching Laboratories at Baghdad Teaching Hospital/Medical City.

Thirty cases were diagnosed as adenocarcinoma of the endometrium, an additional 15 patients diagnosed as to have uterine leiomyoma(fibroid) and who had normal endometrium were taken as a control group. The patient's age ,tumor grading ,depth of myometrial invasion, presence of pelvic and paraaortic LN metstases, vascular invasion ,and the stage of the disease were noted .

The specimens were already fixed in10% formalin , and paraffin embedded. Three sections (4 micron in thickness) were cut from each paraffin block. One section stained with Haematoxylin and Eosin (H&E) stain , and the other two stained with HGF and c-Met immunostaining antibodies using positively charged slides .

RESULTS:

The mean age of the patients with endometrial adenocarcinoma was 58.5years**.**

There was no statistically significant difference between HGF/c-*Met* expression and age, tumor grade, stage , myometrial invasion and vascular invasion. There was a statistical significant correlation between HGF and c-*Met* scores with cases showing no pelvic lymph nodes metastases.

There is a linear increase in HGF and c-*Met* expression in both the diseased and control group.A statistical significant correlation was found in HGF and c-*Met* scores between the diseased group and the control group.

CONCLUSION:

HGF and c-*Met* staining was significantly different between control group and diseased group.

HGF and c-*Met* Showed Linear increase Expression in both diseased and control group .

The c-*Met* is the high-affinity receptor for hepatocyte growth factor.

KEYWORDS: HGF, c-*Met* , endometrial adenocarcinoma.

INTRODUCTION:

Endometrial carcinoma is a primary malignant epithelial tumor, usually with glandular differentiation, arising in the endometrium with the potential to invade and metastasize[(1)] .

Hepatocyte growth factor (HGF) and its receptor c-*M*et have been implicated in uterine development, pregnancy, and endometrial disorders, such as endometriosis and carcinoma.The hepatocyte growth factor (HGF) and its receptor c-*Met* plays an important role in tumor dissemination by activating mitogenic signaling pathways. [(2)].

On binding to the cell surface receptor tyrosine kinase (TK) known as c-*Met*, hepatocyte growth factor (HGF) stimulates mitogenesis, motogenesis, and morphogenesis in a wide range of cellular targets including, epithelial and endothelial cells, hematopoietic cells, neurons, melanocytes, and hepatocytes. These pleiotropic actions are fundamentally important during development, homeostasis, and tissue regeneration. HGF

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signaling also contributes to oncogenesis and tumor progression in several human cancers and promotes aggressive cellular invasiveness that is strongly linked to tumor metastasis[3].

PATIENTS AND METHODS:

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Thirty cases were diagnosed as adenocarcinoma of the endometrium, an additional 15 patients diagnosed as to have uterine leiomyoma(fibroid) and who had normal endometrium were taken as a control group. The patient's age ,tumor grading ,depth of myometrial invasion, presence of pelvic and paraaortic LN metstases, vascular invasion ,and the stage of the disease were noted .

Out of the thirty cases of endometrial carcinoma[15] were found to be stage I , (10) cases were stage II, (3)cases were stage III and (2)cases were stage IV.

Tumor grading : Ten (10 cases) were grade I (well differentiated), (13 cases) were grade II (moderately differentiated) and (7 cases) were grade III
(poorly differentiated).

Myometrial invasion was divided into :No myometrial invasion 3 cases ,equal or less than one half of the myometrial invasion i.e within the inner half 11cases and more than one half of the myometrial invasion i.e the outer half of the uterine wall were 16 cases.

Pelvic lymph node metastases seen in 4 cases, where as 26 cases showed no pelvic lymph node metastases.

Vascular invasion was observed in one case only.

The specimens were already fixed in10% formalin , and paraffin embedded. Three sections (4 micron in thickness) were cut from each paraffin block. One section was stained with Haematoxylin and Eosin (H&E) stain , and the other two stained with HGF and c-Met immunostaining antibodies using positively charged slides .

The source of the specific reagents are as follow: HGF antibody (H2005-08 A), USBiological(USA), c-Met antibody (sc-161) , Santa Cruz Biotechnology , USA, Immunohistochemistery Detection Kit , USBilogical (USA).

Immunohistochemistery procedure:

The slides were incubated over night at 65c in vertical position for deparaffinization then:

Day 1 :

The slides were soaked in Xylene twice ,each time for 15 minutes.

Dehydration by Ethanol alcohol in the following order:100%(I),100%(II), 95%, 90%, 80%, 70% for 5 minutes in each solution then in distilled water for 5 minutes.

The slides were immersed in 0.3% H2O2 for 30 minutes at room temperature

The slides were rinsed by distilled water followed by 1X PBS .

Antigen retrieval was done by immersing the slides in citric buffer jar and put in microwave oven set at 720 watt for 10 minute .

The tissues were circled with pap pen

Incubation of the slides with 1% normal serum was done for 30 minutes at room temperature .

Normal serum dropped off and overnight incubation with HGF and c-MET antibody was performed.

Day 2:

The slides were rinsed 3 times with 1X PBS for 3 minutes each .

Incubation with 100 µl of anti-rabbit IgG(biotin) was done for 20 minutes. step one repeated.

The slides were then incubated with 100 µl of detection solution for 10 minutes. step one repeated again.

Incubation with freshly prepared Development solution for 25 minutes was done.

Hematoxylin stain was used by placing 2-3 drops(20 µl) on the slides for 1 minute followed by rinsing with tab water.

Dehydration was done by soaking in graded series of alcohol :70%, 80%, 90%, 95%, 100%(I), 100%(II) , 3 minute each then Xylene (I),Xylene(II) 10 minute each.

Finally DPX and cover slips were added and slides were ready for scoring.

Quality Control:

Positive control tissue: positive control slides prepared from a tissue known to contain the target antigen against which the primary antibody raised. Placental tissue, which is known to exhibit high levels of HGF and c-*Met* protein, was used as positive control.

Negative control(omitting primary antibody): we followed the same steps in the previously mentioned staining procedure but the step of the primary antibody was escaped.

Expected Results: A positive reaction is indicated by a brown colored precipitate at the specific cytoplasmic and nuclear sites of HGF antigen as in figure 1,while a positive immunoreactivity for c-*Met* was observed in both the cell membrane and the cytoplasm of the adenocarcinoma cells, as well as in that of the normal endometrial gland cells as seen in figure 2.

Immunostaining was independently evaluated by two investigators who had no previous knowledge of the clinical data. In case of different evaluations, the lower score was adopted.

Furthermore HGF and c-Met expression was correlated with the clinico-pathological variables of the tumor by semi quantitative analysis [12], where the counting of positive cells was performed at oil immersion(x1000) as follows:

Score 0, no positively stained tumor cells detected (-).

Score 1, less than one-third positively stained tumor cells i.e. <33.3 %(+)

Score 2, one-third to two thirds positive tumor cells i.e. 33.3%-66.6 %(++)

And score 3, more than two thirds positively stained tumor cells i.e. >66.6%(+++). [12]

Statistical analysis: SPSS version 18 was used for Statistical analysis . Monte carlo chi square test was used for small sample size, where P-value ≤ 0.05 is considered significant and spearman's correlation test with P-value ≤ 0.01 is considered significant.

RESULTS:

The mean age of the patient with endometrial adenocarcinoma was 58.5years with a range 25-75 years while that of the control group was 49.6 years with a range(42-65years).

The study showed that HGF/c-*Met* immunoreactivity was present in all patients with endometrial adenocarcinomas, and only 80% in patients with normal endometrium(Table 1,2,3&4).

Table 1: Frequency table for HGF score in the control group.

HGF score		Frequency	Percent
	0	3	20.0
	+	10	66.7
	++	2	13.3
	Total	15	100.0

Table 2: Frequency table for c-Met score in the control group.

c-Met score		Frequency	Percent
	0	3	20.0
	+	12	80.0
	Total	15	100.0

Table 3: Frequency table for HGF score in the disease group.

HGF score		Frequency	Percent
	+	7	23.3
	++	18	60.0
	+++	5	16.7
	Total	30	100.0

Table 4: Frequency table for c-*Met* score in the disease group.

c-Met score		Frequency	Percent
	+	14	46.7
	++	13	43.3
	+++	3	10.0
	Total	30	100.0

There was no statistically significant difference between both age categories in the control group and diseased group with HGF and c-*Met* scores.

HGF/c-*Met* expressions were seen in the three categories of myometrial invasion, all tumor grades and stages, and vascular invasion ,but there were no significant statistical correlation found between HGF/c-*Met* expressions and myometrial invasion, tumor grade,tumor stage and vascular invasion.

There was a statistical significant correlation between HGF and c-*Met* scores with cases showing no pelvic lymph nodes metastases , where P-value=0.011 and 0.009 respectively as in table 5&6.

Table 5: Cross Tabulation Between HGF score and pelvic lymph node metastases in the 30 cases with endometrial adenocarcinoma.

			HGF score			Total
			+	++	+++	
lymph node metastasis	positive	Count	0	1	3	4
		% within lymph node metastasis	.0%	25.0%	75.0%	100.0%
	negative	Count	7	17	2	26
		% within lymph node metastasis	26.9%	65.4%	7.7%	100.0%
Total		Count	7	18	5	30
		% within lymph node metastasis	23.3%	60.0%	16.7%	100.0%

Monte Carlo test ,P value=0.011

Table 6: Cross Tabulation Between c-Met score and pelvic lymph node metastases in the 30 cases with endometrial adenocarcinoma.

			c-*Met* score			Total
			+	++	+++	
lymph node metastasis	positive	Count	0	2	2	4
		% within lymph node metastasis	.0%	50.0%	50.0%	100.0%
	negative	Count	14	11	1	26
		% within lymph node metastasis	53.8%	42.3%	3.8%	100.0%
Total		Count	14	13	3	30
		% within lymph node metastasis	46.7%	43.3%	10.0%	100.0%

Monte Carlo test ,P value= 0.009

There is a linear increase in HGF and c-*Met* expression in both the diseased and control group. A statistical significant correlation was found in HGF and c-*Met* scores between the diseased group and the control group ; where P-value= 0.0001 for both as in table 7.

Table 7: Cross tabulation between HGF and c-Met scores in the 30 cases with endometrial adenocarcinoma.

			HGF score			Total
			+	++	+++	
cMET score	+	Count	7	7	0	14
		% within cMET score	50.0%	50.0%	.0%	100.0%
	++	Count	0	11	2	13
		% within cMET score	.0%	84.6%	15.4%	100.0%
	+++	Count	0	0	3	3
		% within cMET score	.0%	.0%	100.0 %	100.0%
Total		Count	7	18	5	30
		% within cMET score	23.3%	60.0%	16.7%	100.0%

Monte carlo test ,p-value=0.0001

DISCUSSION:

In the current study the expression of c-*Met* in the primary tumor as assessed by immunohistochemical staining was not correlated with all prognostic factors of patients with endometrial adenocarcinomas. In addition , because almost all of the cases which showed immunoreactivity for c-*Met* also demonstrated positive immunoreactivity for HGF, the colocalization of both HGF and c-*Met* may be a significant factor in the prognostication of endometrial adenocarcinomas. This observation would suggest that an autocrine mode of growth stimulation exists in these tumors[4].

The present study showed a linear increase in the expression of the two markers in both the control and diseased group as well.

HGF was expressed in tissues from a large number of patients with both normal endometrium and endometrial adenocarcinomas, and there was no significant correlation between HGF expression and any prognostic factors except for lymph nodes metastases.

Considering the qualitative and quantitative nature of immunohistochemical staining, it may be useful to investigate HGF expression by other quantitative methods such as Western blot or enzyme-linked immunosorbent assay, to examine

the relationship between HGF and the prognosis of endometrial adenocarcinomas [5].

The immunolocalization of c-*Met* protein to the apical plasma membrane of the epithelial lining of both the normal and malignant glands is most consistent with its being a membrane-bound receptor protein synthesized by the cells[6].

Our study showed no significant statistical correlation between HGF/c-*Met* expression and tumor grade, stage, myometrial invasion, and vascular invasion, that was in agreement with a study by (Bishop EA et al 2011)[7] except for lymph nodes metastases ; where our study showed significant statistical correlation between lymph nodes metastases and HGF/c-*Met* expression.

While a study by (Cem Baykal et al 2002)[8]on uterine cervix carcinomas showed a significant statistical correlation between HGF/c-*Met* expression and tumor grade, stage, stromal invasion, vascular invasion and pelvic lymph node metastases.

In our study, the patients with cancer had more total c-*Met* and HGF expression than those with normal endometrium that was in agreement with(Bishop EA et al 2011)[7]

HGF has a major role in tumor proliferation, migration, invasion, and metastasis through c-*Met* pathway in a variety of cancers. Tumorigenic activity of c-*Met* depends on deregulation of the HGF/c-*Met* signaling pathway that results in phosphorylation and activation of AKT [9].

Although the major HGF-producing cell types are mesenchymal cells, such as fibroblast and vascular smooth muscle cells, both HGF mRNA expression and protein synthesis of HGF have been reported in cells from breast carcinoma and lung carcinoma.[10] In the current study, HGF staining was found in normal endometrial glands and adenocarcinoma cells.

To determine whether the HGF immunereactivity in the normal endometrial glands and adenocarcinoma cells represents the internalized HGF/c-*Met* complex, it may be necessary to study the expression of HGF mRNA [10].

The evaluation of HGF/c-*Met* expression, which promotes the tumor progression, may also be useful in the induction of new anti-tumor drugs to supplement conventional chemotherapy [11].

The correlation between HGF/c-*Met* expression and various clinicopathological factors:

The control group:

In the current study our results showed that c-*Met* immunoreactivity was not present in all patients with normal endometrium(only 80%). , and was found more often in patients with endometrial adenocarcinomas, that was also showen by a study from Japan by Satoshige et al 1998[5] .A study by Toru Furukawa et al 1995[12] on pancreatic cancer showed no immunoreactivity for either HGF or c-*Met* in normal pancreatic duct epithelium However, positive HGF immunostaining was demonstrated in hyperplastic/dysplastic ductal epithelium.

Bishop EA et al 2010[7] showed that c-*Met* and HGF staining were significantly different between control group and diseased group ($p=0.042$, $p<0.001$ respectively), and that was in agreement with the recent study where P- value = 0.001.

Age:

The mean age of patients with endometrial adenocarcinoma in our study was 58.5 years .A study done by Yassoub in 1997 [13], in Baghdad showed that the mean age of patients with endometrial adenocarcinoma was 55.6 years . In 1996 Kalandidi A et al[14] showed that the mean age of patient with endometrial adenocarcinoma was 67 years .

Bishop EA et al 2010[7] showed that high HGF staining in endometrial adenocarcinoma did correlate to age over 65years ($p=0.05$) ,while the current study showed no statistically significant difference between both age categories in the control and diseased groups with HGF and c-*Met* scores.

Myometrial Invasion:

The present study showed no significant statistical correlation between HGF and c-*Met* expressions with myometrial invasion; this is in concordance with the results by Bishop EA et al 2010[7] and Satoshige Wagatsuma et al[5].

Tumor grade:

Bishop EA et al 2010 [7]found that there were no significant statistical correlation between HGF and c-*Met* expression and tumor grade, this is agreed with the recent study.

Wagatsuma S. et al[5] showed that HGF/c-*Met* expression were significantly correlated with surgical Stage III and IV, histologic Grade 3, and myometrial invasion > 1/2.

Tumor stage:

A study by Antonio Belfiore et al 1997[15] showed that *Met*/HGF-R expression was unrelated to histological grade, tumor size, extrathyroid invasion, metastatic lymph nodes, or patient age, and was associated only with histological evidence of vascular invasion in thyroid papillary carcinoma.

Our study showed no significant statistical correlation between HGF and c-*Met* expression with tumor stage .

Pelvic lymph nodes metastases:

There were a statistical significant correlation between HGF and c-*Met* scores with cases showing no pelvic lymph nodes metastases.

Cem Baykal et al [8] in their study on uterine cervix carcinomas showed over expression of HGF and c-*M*et in cases of lymph node metastasis and the presence of metastatic lymph node and immunopositivity for c-*M*et are significantly correlated with overall survival ,while the study by Bishop EA et al[7]showed no significant correlation between HGF and c-*M*et with the lymph node metastasis.

A study looking at the power of c-*Met* in detecting lymph node metastases in head and neck squamous cell carcinomas revealed that c-*Met* gene product is a valuable marker for detection of occult tumor cells in lymph nodes [16].

Vascular invasion: A study by Antonio Belfiore et al 1997[15] on Papillary thyroid carcinoma showed that carcinoma with negative/low *Met*/HGF-R expression were associated with vascular invasion and distant metastases .

The correlation between HGF and c-*Met* expression:

The c-*Met* is the high-affinity receptor for hepatocyte growth factor (HGF, also known as scatter factor; Bottaro et al 1991) [17] ,c-*Met* is the only receptor for HGF and that HGF is the sole c-*Met* ligand (Birchmeier et al 2003).[18] Epithelial cells respond in vitro to HGF/c-*Met* signaling by "scattering" with a concomitant increase in their motility and ability to invade collagen matrices (Weidner et al.,1990) [19].

It is well established that HGF functions as a potent growth factor for liver development, being produced by mesenchymal cells and acting in a paracrine manner on adjacent c-*Met*-expressing epithelial hepatocytes. (Sonnenberg et al 1993) [20].

It was previously concluded that both molecules are mis- and over- expressed in HNSCC and probably induce the program of invasive growth in HNSCC [16].

Hong-Lin Cheng et al [21]studied the prognostic importance of *Met* compared with P53 in the transitional cell carcinoma of the urinary bladder, found that HGF/*Met* signaling plays an even more important role in the carcinogenesis of human bladder.

There is a substantial body of experimental evidence supporting an oncogenic role for the HGF/*Met* signaling pathway. High levels of *Met* expression have been correlated with the metastatic spread of tumors and poor survival in patients with breast carcinoma, extrahepatic biliary tract cancer, gastric cancer,endometrial carcinoma, hepatocellular carcinoma, colorectal cancer, and renal cell carcinoma.[22]

CONCLUSION:

HGF and c-*Met* staining was significantly different between control group and diseased group.

HGF and c-*Met* Showed Linear increase Expression in both diseased and control group .

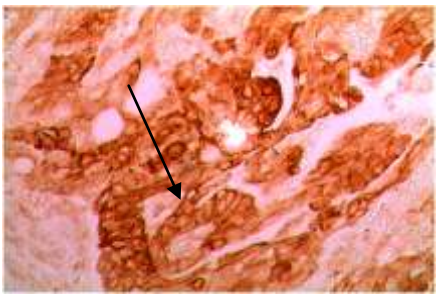

Figure 1: Well differentiated endometrial adenocarcinoma immunostained with HGF immunomarker showing positive immunoreactivity indicated by a brown colored precipitate at the specific cytoplasmic and nuclear sites of HGF antigen(arrow).(x400).

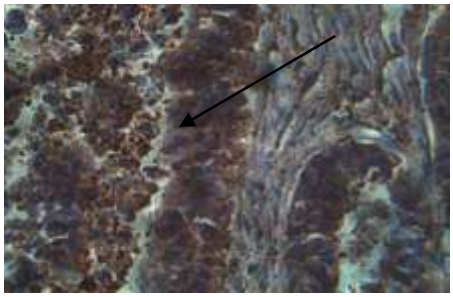

Figure 2: The same picture seen in figure 15 showing positive immunoreactivity for c-*Met* observed in both the cell membrane and the cytoplasm of the adenocarcinoma cells(arrow).(x400).

REFERENCES:

1. Surveillance, Epidemiology, and End Results (SEER) Program. SEER Database: Incidence - SEER 9 Regs Public-Use. National Cancer Institute, DCCPS, Surveillance Research Program .U.S.National Institutes of Health/www.cancer.gov,2008.
2. Kumar V., Abbas AK., Fausto N., Aster JC.: Robbins and Cortan Pathologic Basis of disease 8th edition, 2010:87-92.
3. Grierson I, Heathcote L, Hiscott P, Hogg P, Briggs M, Hagan S. Hepatocyte Growth Factor/Scatter Factor in the Eye. *Progress in Retinal and Eye Research.* 2000;19:779-802.
4. Comoglio PM. Pathway specificity for MET signaling. *Nature Cell Biology* 2001;3:161-62.
5. Wagatsuma S, Konno R, Sato S, Yajima A. : Tumor angiogenesis, hepatocyte growth factor, and c-Met expression in endometrial carcinoma. *Cancer.* 1998;1;82:520-30.
6. Peruzzi B. and Bottaro DP. : Targeting the c-*Met* Signaling Pathway in Cancer . *Clin Cancer Res* 2006; 12: 3657.
7. Bishop EA, Lengyel ER, Yamada SD, Montag A, Temkin SM.: The expression of hepatocyte growth factor (HGF) and c-Met in uterine serous carcinoma. *Gynecologic Oncology* 2010;121:218–23.
8. Baykal C, Atakan Al, Yuce K, Ayhan A.: Overexpression of the c-Met/HGF receptor and its prognostic significance in uterine cervix carcinomas. *Gynecologic Oncology* 2003;88:123–29.
9. Xiao GH, Jeffers M, Bellacosa A.: Anti-apoptotic signaling by hepatocyte growth factor/Met via the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pathways. *Proc Natl Acad Sci USA* 2001;98:247–52.
10. Kim S, Lee UJ, Kim MN et al :"MicroRNA miR-199a regulates the MET proto-oncogene and the downstream extracellular signal-regulated kinase 2 (ERK2)".J. Biol. Chem. 2008;283:18158–66.
11. Trusolino L, Comoglio PM. Scatter-Factor and Semaphorin Receptors: Signalling for Invasive Growth. *Nature Reviews Cancer* 2002;2:289-300.
12. Furukawa T , Duguid WP, Kobari M, Matsuno S, Tsao MS: Hepatocyte Growth Factor and Met Receptor Expression in Human Pancreatic Carcinogenesis .*American Journal of Pathology*. 1995;147:889-95.
13. Yassoub N : Endometrial Carcinoma and its Precancerous Conditions(Endometrial Hyperplasia).A Theses Submitted to ICMS Pathology , 1997.
14. Kalandidi A, Tzonou A, Lipworth L, Gamatsi I, Filippa D, Trichopoulos D : A Case Control Study of Endometrial Cancer in Relation to Reproductive , Somatometric and Life-style Variables .*Oncology* 1996;53:354-59.
15. Belfiore A, Gangemi P, Costantino A et al : Negative/Low Expression of the Met/Hepatocyte Growth Factor Receptor Identifies Papillary Thyroid Carcinomas with High Risk of Distant Metastases, *Journal of Clinical Endocrinology and Metabolism* 1997;82:2322-28.
16. De Herdt MJ, Baatenburg de Jong RJ: HGF and c-MET as potential orchestrators of invasive growth in head and neck squamous cell carcinoma. *Front Biosci.* 2008;13:2516-26.
17. Bottaro DP, Rubin JS, Faletto DL et al : "Identification of the hepatocyte growth factor receptor as the met proto-oncogene product".*Science* 1991;251:802–4.
18. Birchmeier C, Birchmeier W, Gherardi E, Vande Woude GF. "Met, metastasis, motility and more". *Nat. Rev. Mol. Cell Biol. (December* 2003;4:915–25.

19. Weidner KM, Di Cesare S, Sachs M, Brinkmann V, Behrens J, Birchmeier W. "Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis". *Nature* 1996;384:173–76.

20. Sonnenberg E, Meyer D, Weidner KM, Birchmeier C : Scatter factor/hepatocyte growth factor and its receptor, the c-Met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. *The Journal of Cell Biology* 1993;123:223-35.

21. Cheng HL, Trink B, Tzai TS et al : Overexpression of c-met as a Prognostic Indicator for Transitional Cell Carcinoma of the Urinary Bladder: A Comparison With p53 Nuclear Accumulation. *Journal of Clinical Oncology* 2002;20:1544-50.

22. Di Renzo MF, Narsimhan RP, Olivero M,: Expression of the Met/HGF receptor in normal and neoplastic human tissues. *Oncogene*, 1991;6:1997-2003.