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## Evidence for Heightened Hexokinase II Immunoexpression in Hepatocyte Dysplasia and Hepatocellular Carcinoma

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### Abstract

**Background**—Normal hepatocytes exhibit low-affinity hexokinase (glucokinase [HKIV]), but during oncogenesis, there is a switch from HKIV to HKII expression. The aims of this study were to compare the immunoexpression of HKII in non-dysplastic cirrhosis (NDC), liver cell change/dysplasia in cirrhosis (LCD), HCC, and normal liver control tissues, and to correlate HKII expression with clinical and histopathological parameters.

**Design**—Immunohistochemistry was performed on a liver cancer progression tissue array consisting of specimens from explants with cirrhosis, including 45 tissue samples with HCC, 108 without HCC, 143 with LCD, and 8 normal liver control tissues. HKII expression was quantified as positive pixel counts/square millimeter (ppc/mm<sup>2</sup>) by image analysis.

**Results**—There was a stepwise increase in HKII level from normal liver tissue to NDC, to LCD, and to HCC ( $p = 0.001$ ). HKII levels were significantly higher in areas of LCD versus NDC ( $p = 0.001$ ), and in LCD and HCC versus NDC ( $p = 0.007$ ). HKII levels were similar in LCD and HCC ( $p = 0.124$ ). HKII levels were higher in grade 2–4 versus grade 1 HCCs ( $p = 0.044$ ), and in pleomorphic versus non-pleomorphic HCC variants ( $p = 0.041$ ). Higher levels of HKII expression in LCD and HCC versus NDC and in higher tumor grade remained significant in multivariate analysis.

**Conclusions**—Higher levels of HKII immunoexpression in LDC and HCC compared with NDC suggest that upregulation of HKII occurs during the process of hepatocarcinogenesis in humans. In HCC, higher levels of HKII are associated with more aggressive histological features.

### Keywords

Hepatocellular carcinoma; Liver cell change/dysplasia in cirrhosis; Non-dysplastic cirrhosis; Hexokinase II immunohistochemistry; Tissue microarray; Image analysis

### Introduction

Data from the SEER program registries showed that the incidence of liver cancer increased between 1975 and 2005 at an annual percent change of 4.1 % in men and 3.8 % in women [1]. Hepatocarcinogenesis results from a series of pro-oncogenic molecular events and morphological alterations during the course of liver disease progression. In most cases, the sequence of hepatocarcinogenesis includes the development of cirrhosis, followed by dysplasia, and HCC [2]. An increase in hepatitis C virus (HCV) cirrhosis has played a major role in the rise in HCC and now accounts for 25–50 % of cases in the USA [3, 4]. There is growing evidence that insulin resistance and inflammatory factors implicated in the development of nonalcoholic fatty liver disease (NASH) can promote hepatocarcinogenesis [5–10]. Given the increasing incidence of HCC, it is critical to better understand molecular events associated with tumor development in order to identify effective biomarkers and therapeutic targets.

In the absence of liver disease, normal adult hepatocytes express only the low-affinity glucokinase (HKIV) for the regulation of glucose homeostasis in the feeding and fasting states [11]. A transformation from HKIV to high-affinity HKII expression allows for the high glycolytic rates that occur in anaerobic metabolism during tumorigenesis and tumor

progression [12–14]. Levels of mitochondrial hexokinase can be enhanced up to 200-fold in metabolically active malignant cells in comparison with normal tissues [15].

HKII was characterized in benign versus malignant hepatocytes in rat immunohistochemistry [16] and DNA genomic studies [17]. HKII is not expressed in the majority of adult human normal tissues, but is upregulated by oncogenic signaling and insulin. HKII was found to be enhanced in a number of human cancers including HCC [18–22]. In HCC, there is an isoform change from the low-affinity glucokinase to the high-affinity HKII. The expression of HKII in HCC cells distinguishes them from normal hepatocytes. Recent observations in humans have shown an association between increased HKII immuno-expression and higher HCC tumor grade, stage [23, 24], and poorer patient survival [24, 25]. However, it is not known when HKII expression becomes up-regulated in the sequence of hepatocarcinogenesis. The goals of this study were to compare the immunoexpression of HKII in non-dysplastic cirrhosis (NDC), liver cell change/dysplasia in cirrhosis (LCD), HCC, and normal liver control tissues, and to correlate HKII level with clinical and histopathological parameters.

## Materials and Methods

### Subjects

Subjects consisted of 153 patients who underwent liver transplantation at the University of Illinois Hospital and Health Sciences System between 2004 and 2012 and 8 normal controls. All subjects had available clinical data and archived liver tissue for immunohistochemistry (IHC). Histological sections of cirrhotic liver explants with and without LCD and HCC were examined, and corresponding areas of the tissue blocks were sampled such that the total number of specimens exceeded the number of explants. Forty-five specimens had cirrhosis with HCC, 108 had non-dysplastic cirrhosis (NDC) without HCC, and 143 of the cases had areas of LCD including 98 cases without HCC and 45 with HCC. Eight normal liver control samples were obtained from uninvolved, non-diseased liver tissues of benign resection specimens for hemangioma and focal nodular hyperplasia. The demographic and clinical features of all 153 subjects in the study population were obtained from review of electronic medical records.

### Gross and Microscopic Evaluation of Liver Tissues

Explants were grossly examined and sectioned at 0.5-cm intervals according to the segmental distribution of the liver. The size, location, and number of tumor(s) were recorded. Samples were obtained for microscopy according to published guidelines [26], and sections were histopathologically evaluated by a pathology team (RC, GG, RP). Dysplastic hepatocytes were defined by the presence of abnormal nuclear size, exhibiting small cell change or large cell change [27–30] bizarre hyperchromatic or occasionally multiple nuclei; distribution in clusters, groups, or occupying a whole nodule; and absent liver cell plate expansion [31, 32]. Malignant hepatocytes consistent with HCC were identified by the presence of thickened liver cell plates, increased nucleocytoplasmic (N/C) ratio, atypical vesicular nuclei, and when present, bile production [33]. The tumors were evaluated for tumor type, grade, and stage following the guidelines of the AJCC [34]. Tumor pattern was

classified as pseudoglandular, trabecular, solid, or combined [35]. Tumor variants were designated as non-pleomorphic (usual variant), pleomorphic, and combined according to criteria outlined by Ferrell [36].

### Tissue Microarrays (TMA)

The adequacy, quality, and structural integrity of the paraffin-embedded tissue blocks were assessed and verified prior to inclusion in the study. Explant liver samples from HCC and cirrhosis cases provided ample tissue for construction of arrays. The tissue microarrays were designed and constructed based on prescribed practices [37] using the Beecher manual tissue arrayer.

Hematoxylin and eosin-stained adjacent tissue sections were reviewed and used to identify areas of NDC and LCD in liver explants with and without hepatocellular carcinoma and HCC lesions in liver explants with HCC. Cores from archived normal liver specimens provided non-disease liver controls. Donor core tissues with 0.6 mm diameter were obtained from corresponding regions in the donor paraffin block and embedded in a recipient block. A single donor core tissue from each lesion consisting of NDC, LCD, and when present, HCC, was sampled across a patient's liver explant. The donor cores from all subjects were embedded in a tissue microarray (TMA) recipient block. A total of two TMA recipient blocks were prepared, one consisting of liver tissue cores obtained from liver explants of subjects without HCC (2 donor cores were obtained per subject representing NDC and LCD), and another TMA contained liver tissue cores of patients with HCC (3 donor cores were taken from each subject, representing NDC, LCD, and HCC). Each of the tissue microarray cores was histologically examined to document and record tissue-sampling adequacy and diagnoses. Four-micron sections from the TMAs were placed on positively charged glass slides for IHC.

### Immunohistochemistry (IHC)

Immunohistochemical staining was performed using the avidin–biotin–peroxidase complex method [38]. Formalin-fixed, paraffin-embedded histological sections were de-waxed in xylene and then rehydrated by passage through graded alcohol. The tissue arrays were rinsed and then incubated with a solution of 0.3 % hydrogen peroxide, followed by incubation with 2 % BSA and then 10  $\mu$ M sodium citrate buffer at pH 6.0. Anti-hexokinase II (C64G5) rabbit mAb (Cell Signaling<sup>®</sup>), 300  $\mu$ L, was added to slides and maintained at 4 °C overnight in a humidified chamber. Sections were washed with PBS and then incubated with a 1: 300  $\mu$ L dilution of biotinylated antibody to rabbit, followed by washing and incubation with a 1:1,000 dilution of avidin and biotin in PBS. Avidin–biotin complex was washed off three times in buffered solution for 5 min each. 100–400  $\mu$ L DAB was added, and slides were immersed in dH<sub>2</sub>O. Sections were dehydrated by incubation in 95 % ethanol twice for 10 s each. This was repeated in 100 % ethanol twice for 10 s each. Additional washes with xylene were performed twice for 10 s each time. Slides were counterstained with hematoxylin and washed with dH<sub>2</sub>O twice for 5 min, and coverslips were mounted. HK II signals were described as concentrating primarily within the external mitochondrial membrane, resulting in a granular pattern of cytoplasmic staining [39].

## Digital Imaging and Image Analysis

The Aperio system (ScanScope CS) was used for digital imaging and image analysis of HKII immunostained slides. Image analysis was performed using ImageScope® software from Aperio. An inclusion pen for selecting image analysis outputs was used to outline pertinent tissue samples representing hepatocytes. Non-hepatocyte tissues were isolated with an exclusion device. Each of the cores was histologically examined for the verification of sample adequacy and diagnoses. An HKII immuno-level was measured from each core, representing NDC, LCD, and when present, HCC and recorded in an excel database file. Scoring of hexokinase II was confined to the cytoplasm consistent with its characteristic staining features as described in published immunohistochemical validation studies for HKII [39]. Image analysis outputs of HKII immuno-levels were expressed in  $\text{ppc}/\text{mm}^2$ .

## Analysis

Data analysis was performed using SPSS (SPSS, Inc., version 17.0, Chicago, IL) and SAS 9.2 (Cary, NC). Descriptive statistics were used to evaluate for normalcy of data and for erroneous entries. For independent observations, normally and non-normally distributed continuous variables were analyzed using the Student *t* test and the Mann–Whitney *U* test, respectively. Pearson chi-square testing was used to compare categorical variables. Overall nonparametric Friedman tests were used to compare the ranks [40] of HKII scores among correlated samples within the same patients to examine the progression of staining over disease stage. We also conducted paired statistical analysis using nonparametric Wilcoxon signed-rank tests to perform pairwise comparison. ANOVA was used to evaluate the mean differences in HKII scores among different groups defined by tumor grade, tumor variant, tumor stage, and African American versus non-African American ethnicities. Multiple linear regression models were used to adjust for important covariates and potential confounders.

## Results

### Demographic and Clinical Features of Subjects

Clinical data are summarized in Table 1, stratified by HCC status. Subjects with HCC were older than those without HCC ( $p = 0.001$ ). There were no significant differences in gender, ethnicity, cause of liver disease, or body mass index (BMI), between patients with and without HCC.

### Physical Characteristics of Hepatocellular Carcinoma

The liver explants had a mean of  $1.8 \pm 1.2$  tumor lesions with a mean size of  $3.85 \pm 2.88$  cm. Morphological patterns were pseudoglandular (18.6 %), trabecular (46.5 %), solid (18.6 %), and combined (16.3 %). The distribution of tumor grades was grade 1 (32.6 %), grade 2 (37.2 %), and grades 3 and 4 (30.2 %). The tumor variants were divided as usual (39.5 %), clear cell (2.3 %), pleomorphic (11.6 %), combined pleomorphic (11.6 %), and combined non-pleomorphic (34.9 %) variants of HCC. The tumor stages were classified as stage I (50 %), stage II (38.9 %), stage III (8.3 %), and stage IV (2.8 %). The average tumor stage at the time of surgery was  $1.64 \pm 0.76$ . Lymphovascular invasion was identified in 9.6 % of the tumor cases.

## Immunohistochemistry

The staining characteristics of HKII are illustrated in Fig. 1. Staining for HKII was readily identified in all NDC, LCD, and HCC samples, was predominantly localized to the cytoplasm, and was characterized as finely granular. The mean level of HKII was  $36.67 \pm 15.20$  ( $n = 8$ ) in normal controls,  $44.18 \pm 32.71$  ( $n = 108$ ) in NDC,  $59.94 \pm 39.86$  ( $n = 143$ ) in LCD, and  $64.42 \pm 34.89$  ( $n = 45$ ) in HCC ( $p = 0.001$ ). The box plot in Fig. 2 shows ranges and mean hexokinase II immuno-levels in normal liver control ( $n = 8$ ) and non-dysplastic cells ( $n = 108$ ), liver cell dysplasia ( $n = 143$ ), and hepatocellular carcinoma ( $n = 45$ ).

HKII levels were higher in areas of LCD versus NDC with a mean difference of  $13.44 \pm 23.30$  ( $p = 0.001$ ) ( $n = 143$ ) in all subjects with cirrhosis without and with HCC. Higher HKII expression was observed in areas of LCD versus NDC in patients with cirrhosis without HCC ( $p = 0.001$ ) ( $n = 98$ ) and in cases with cirrhosis with HCC ( $p = 0.007$ ) ( $n = 45$ ). In patients with HCC, HKII levels were greater in areas of LCD and HCC than in NDC (mean difference  $15 \pm 23.93$ ) ( $p = 0.007$ ). There was no significant difference in HKII expression between areas of LCD and HCC (mean difference  $7.60 \pm 42.43$ ) ( $p = 0.124$ ).

HKII expression was higher in grade 2–4 HCC ( $71.40 \pm 32.66$ ) versus grade 1 HCC ( $48.96 \pm 35.84$ ) ( $p = 0.044$ ), and in pleomorphic ( $85.22 \pm 35.85$ ) compared with non-pleomorphic HCC variants ( $57.03 \pm 25.33$ ) ( $p = 0.041$ ). Stage II–IV HCC had higher HKII levels ( $71.95 \pm 44.90$ ) than stage I HCC ( $48.50 \pm 33.07$ ) ( $p = 0.047$ ). In multivariate analysis adjusting for age, gender, ethnicity, cause of liver disease, BMI, and lymphovascular invasion, higher levels of HKII expression remained significantly associated with LCD and HCC versus NDC ( $p = 0.007$ ) and higher (grades 2–4) versus lower tumor grade HCC ( $p = 0.044$ ).

An exploratory analysis was performed to evaluate for associations between clinical factors and HKII expression. African Americans (AA) had higher levels of HKII expression compared with non-AA (NAA) in NDC (AA  $62.10 \pm 41.17$  vs. NAA  $36.45 \pm 28.15$ , ( $p = 0.002$ ) and in LCD (AA  $72.97.10 \pm 46.16$  vs. NAA  $54.80 \pm 36.77$ ,  $p = 0.020$ ). HKII levels in HCC were similar between AA ( $75.63 \pm 46.32$ ) and NAA  $59.64 \pm 31.56$ ) ( $p = 0.152$ ). There is no significant relationship between race and tumor grade, tumor stage, or tumor variant. There was higher level of HKII in (DM+) in comparison with (DM-) in dysplastic lesions of the explanted livers of subjects without HCC ( $p = 0.018$ ). There were no significant associations between DM and HKII levels in HCC lesions of subjects with HCC.

## Discussion

The novel finding of this study was that HKII immunoexpression was similar in liver cell change/dysplasia and HCC and greater in liver cell dysplasia and HCC than in cirrhotic tissue without dysplasia. The results indicate that HKII level increases prior to the development of HCC and suggest that HKII expression should be evaluated as a potential biomarker for HCC risk in patients with cirrhosis. Hexokinase II has been identified in the glycolytic pathway that occurs in the hypoxic environment of rapidly growing tumors including HCC [13, 41]. Data from the current study raise the compelling question of whether HKII is involved in the transition from cirrhosis to dysplasia, perhaps by providing an energy source for proliferation of dysplastic hepatocytes while inhibiting apoptosis [42,

43] Mechanistic studies are needed to assess for a causal relation between HKII expression and progression to HCC.

The strengths of this study include well-characterized liver tissue samples from a robust cohort of patients at risk for hepatocellular carcinoma and the use of tissue arrays and image analysis. Application of tissue arrays in research has been reported to provide an efficient and cost-effective means to evaluate immunohistochemistry (IHC) in large numbers of cases [44–46]. The specimens were stained simultaneously under the same conditions, reducing the possibility that inter-batch differences in staining technique impacted the results. In contrast to previous works that reported HKII expression in only 45–56 % of samples [24, 25], the current study identified HKII expression in all 45 HCC specimens with an average value of 64.42 ( $\pm$ 34.89) ppc/mm<sup>2</sup>. The differences between the results cannot be explained by variation in antibody or IHC methodology. Here, the use of image analysis provided an objective and sensitive method to quantify immunohistochemical staining that has not been used in previous studies of HKII.

The retrospective, cross-sectional design that limits the findings to associations is a weakness of the current study. Moreover, immunohistochemistry provides information regarding HKII expression, but molecular studies identifying mutational alterations of genes and transcription factors that control HKII over-expression are needed. Sampling adequacy is a frequently raised concern in tissue microarray studies. Protein expression attained from a single 0.6-mm tissue micro-array core has been shown to correlate with the results obtained from whole mount sections. In a study designed to validate the use of tissue microarray as a tool for molecular profiling of cancer biomarkers, up to 95 % concurrence of protein expression from 97 breast tumors was identified between single 0.6 mm cores and whole mount sections, yielding a strong statistical Kappa value of  $>0.90$  [47].

Another item that merits discussion is the consequential upregulation of genes involved in glucose metabolism during adaptation to hypoxic conditions [48]. Molecular studies addressing the role of genes regulating glucose metabolism in tumorigenesis and tumor expansion are appealing as investigators have claimed that hypoxia fuels hepatocellular carcinoma expansion by way of HKII upregulation [48]. However, it is unlikely that HKII expression is induced only in response to hypoxia. Normal adult hepatocytes do not express HKII and only express glucokinase (HKIV) [49]. It is well established that in HCC, there is switch from glucokinase to HKII. HKII is a high-affinity hexokinase, which is required for the high metabolic demands of cancer cells [11–14]. In fact, all HCC cell lines do not express glucokinase and only show HKII in normoxic conditions [50].

While we found a significantly higher level of HKII in (DM+) in comparison with (DM–) in dysplastic lesions of the explanted livers of patients without HCC ( $p$  value = 0.018), there were no significant associations between DM and HKII levels in HCC lesions. HKII is known to be upregulated by insulin [51]. The low number of patients with (NAFLD)/cryptogenic cirrhosis in this series may contribute to one's inability to detect differences in HKII levels between this group and other disease categories.

A number of studies have found that African Americans have an increased risk of HCC compared with others [52–54]. We therefore performed an exploratory analysis to compare HKII expression by race. Interestingly, African Americans had significantly higher levels of HKII in non-dysplastic and dysplastic hepatocytes in cirrhosis than other ethnic/racial groups. While we cannot exclude the possibility that these findings are due to confounding factors, the data provide a rationale for further investigations to assess for racial differences in HKII expression in the development of HCC.

In this study, higher levels of HKII were found to be associated with more aggressive histological features of HCC. Consistent with recent publications, we noted HKII immunoexpression was more pronounced in poorly differentiated, pleomorphic, and in higher stage HCC [23–25], thereby adding to the evidence that HKII is a likely marker for increased metabolic activity that occurs in HCC in humans. The potential use of HK II inhibitors for the treatment of advanced HCCs was described in a mouse tumor model [43], and the question remains whether HKII provides an attractive target for the development of new agents for the treatment of HCC in humans.

In conclusion, HKII expression was higher in HCC with more aggressive histological features and in more advanced-stage disease. Further, the level of HKII in liver cell dysplasia in cirrhosis was similar to that observed in HCC and greater than the degree of expression found in non-dysplastic cirrhosis. Our findings show that HKII levels rise earlier than the occurrence of hepatocellular carcinoma, providing basis for evaluating HKII as a potential biomarker for HCC risk in patients with cirrhosis.

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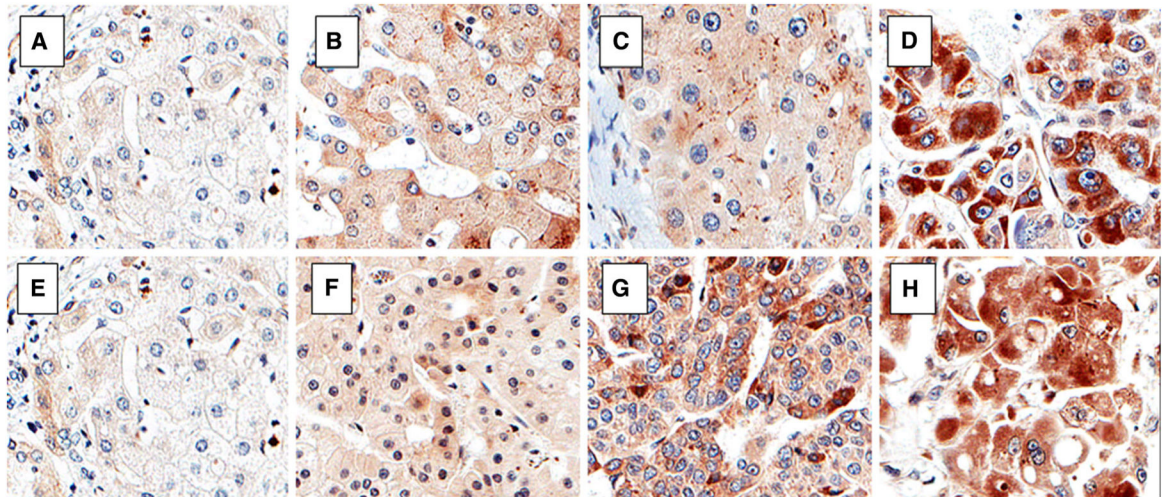
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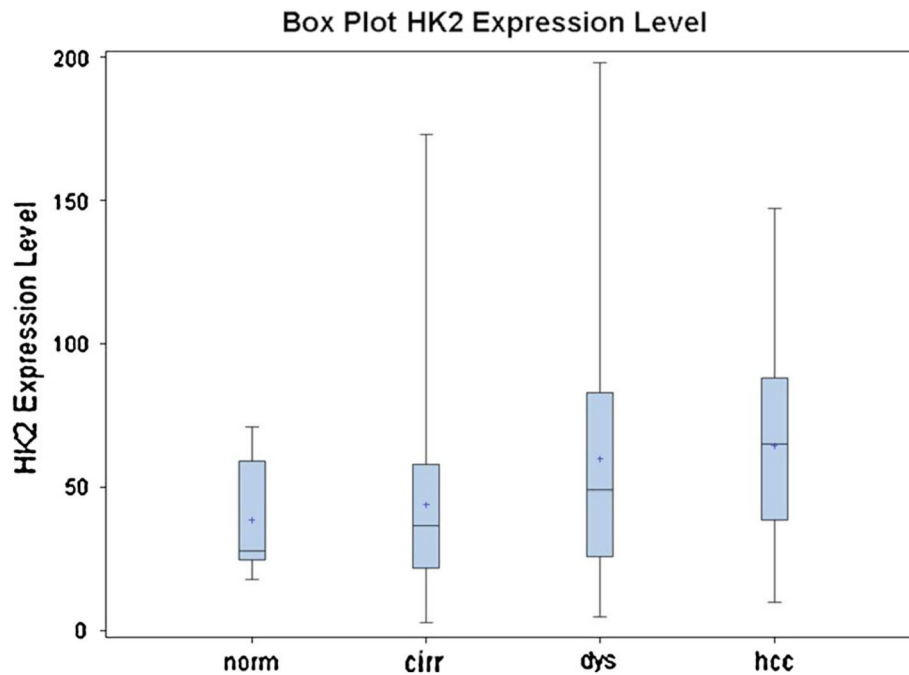
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**Fig. 1.**

Hexokinase II immunorexpression in normal liver, cirrhosis, dysplasia, hepatocellular carcinoma, and advancing tumor grades. Hexokinase II immunorexpression is cytoplasmic. The *upper panel* shows images from a subject's liver explant with a stepwise increase in HKII immunorexpression from non-dysplastic cirrhosis (**b**), to hepatocyte dysplasia (**c**), and hepatocellular carcinoma (HCC) (**d**) in comparison with a normal liver (**a**). The *lower panel* displays sections from various explants with increasing HKII immunorexpression from well (**f**), to moderately differentiated (**g**), to poorly differentiated HCC (**h**), in comparison with normal liver (**e**) ( $\times 20$ )



**Fig. 2.**

*Bar and whiskers box plot illustrating pairwise raw data points of Hexokinase II Immunoeexpression levels in positive pixel counts/mm<sup>2</sup> in normal liver control ( $n = 8$ ) and non-dysplastic cells ( $n = 108$ ), liver cell dysplasia ( $n = 143$ ), and hepatocellular carcinoma ( $n = 45$ ) Obtained from subjects with fibrosis stage IV/0-IV liver explants ( $p = 0.001$ ). Overall, there is a stepwise increase in mean HKII level (denoted as \* in the plot) in positive pixel count ppc/mm<sup>2</sup> in cirrhosis, to dysplasia, to HCC. Paired analysis by Wilcoxon signed-rank test for two samples within the same patient and nonparametric Friedman test for multiple samples within the same patient was used to examine the progression over disease stage*

**Table 1**

Clinical features of liver transplant patients with and without hepatocellular carcinoma (HCC)

Clinical features	Subjects without HCC ( <i>n</i> = 108)	Subjects with HCC ( <i>n</i> = 45)	<i>p</i> value
Mean age in years (SD)	50.02 (±8.98)	55.98 (±11.5)	<b>0.001</b>
Gender (%)			0.420
Female	38.0	31.9	
Male	62.0	68.1	
Ethnicity (%)			0.604
African American	25.2	21.3	
Non-Hispanic Caucasian	37.4	46.8	
Hispanic	32.7	23.4	
Other (%)	4.7	8.5	
Etiology (%)			0.204
Viral (HBV, HCV)	53.7	57.8	
EtOH	23.1	17.8	
NASH/cryptogenic	11.1	6.7	
Other	12.0	17.0	
Mean body mass index (BMI kg/m <sup>2</sup> )(SD) at transplant	30.37 (±7.18)	28.44 (±6.94)	0.069
Diabetes (DM) status (%)	23.1 DM (?)	22.2 DM (?)	0.306

Pairwise variables for mean age in years, gender % (male, female), ethnicity % (African American, non-Hispanic Caucasian, Hispanic, and other race), etiology % (viral, ethanol, NASH/cryptogenic, other etiology), and diabetes status % (positive, negative). All pairwise variables *p* value were not significant, except for age, *p* value = 0.001

Bold value is statistically significant (*p* < 0.05)