

RESEARCH ARTICLE

Human equilibrative nucleoside transporter-1 and deoxycytidine kinase can predict gemcitabine effectiveness in Egyptian patients with Hepatocellular carcinoma

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Abstract

Background: Several biomarkers of gemcitabine effectiveness have been studied in cancers, but less so in hepatocellular carcinoma (HCC), which is identified as the fifth most common cancer worldwide. Investigation of human equilibrative nucleoside transporter-1 (HENT-1) and deoxycytidine kinase (DCK), genes involved in gemcitabine uptake and metabolism, can be beneficial in the selection of potential cancer patients who could be responding to the treatment.

Aim: To study HENT-1 and DCK gene expression in HCC patients with different protocols of treatment.

Methods: Using real-time PCR, we analyzed expression levels of HENT-1 and DCK genes from peripheral blood samples of 109 patients (20 controls & 89 HCC patients) between March 2015 and March 2017. All the 89 HCC patients received the antioxidants selenium (Se) and vitamin E (Vit.E) either alone (45 patients) or in combination with gemcitabine (24 patients) or radiofrequency ablation (RFA) (20 patients).

Results: There was a significant increase in HENT-1 expression levels in HCC patients treated with Se and Vit.E alone as compared to controls ($P < .0001$), while there was no significant difference between HCC patients treated with gemcitabine or RFA as compared to controls. In contrast, expression of DCK was significantly increased in all groups of HCC patients as compared to controls ($P < .0001$).

Conclusions: HENT-1 and DCK mRNA expressions are important markers of HCC and for GEM effect and GEM sensitivity in patients with HCC. This could be beneficial in the selection of HCC patients sensitive to gemcitabine to avoid subjecting resistant patients to unnecessary chemotherapy.

KEYWORDS

deoxycytidine kinase, gemcitabine, hepatocellular carcinoma, human equilibrative nucleoside transporter-1

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1 | INTRODUCTION

HCC is the fifth most common cancer worldwide and the second most common cause of cancer mortality.¹ HCC is unique in that it largely occurs within an established background of chronic liver disease and cirrhosis.² Major causes of cirrhosis in patients with HCC include hepatitis B, hepatitis C, alcoholic liver disease, and possibly nonalcoholic steatohepatitis.³

Because of the aggressive nature of HCC, which results in an overall poor prognosis and short life expectancy, the appropriate choice of effective treatment options is crucial for positive therapeutic outcome. Although there are multiple treatment options available for HCC either pharmacological or non-pharmacological,^{4–9} the average life expectancy after diagnosis of clinically apparent HCC is less than 12 months.¹⁰ Therefore, ineffective treatment outcomes, such as those caused by resistance to chemotherapy, directly affect patients' survival.

Among cancer chemotherapeutic treatments, gemcitabine is widely used as an anti-neoplastic medication that exerts its cytotoxic effect intracellular with documented broad-spectrum antitumor activity in almost all major localizations of cancer.¹¹ Specifically, gemcitabine has been a standard chemotherapy drug for inoperable pancreatic cancer.¹² Because liver is tissue homologous to the gallbladder and pancreas, systemic chemotherapy in advanced or inoperable HCC has drawn lessons from the chemotherapy of other digestive tract cancers. Currently, gemcitabine-based chemotherapy regimens are used in advanced HCC.¹³

If gemcitabine could be appropriately and selectively administered to patients with gemcitabine sensitivity based on the expression of genes in the tumor, maximal chemotherapy efficacy could be achieved without subjecting gemcitabine-resistant patients to unnecessary side effects.¹⁴ Recent investigations using cell lines or surgical specimens have revealed that the expression of several genes may be predictors of gemcitabine efficacy in gemcitabine-treated patients.

Such gemcitabine efficacy predictor genes include HENT-1, the major mediator of gemcitabine uptake in human cells,¹⁵ and DCK, gemcitabine metabolism-related enzyme.¹⁶ Indeed,¹⁷ and¹⁸ demonstrated that levels of expression of these genes correlated with gemcitabine sensitivity in patients with pancreatic ductal carcinoma.

In order to reach their intracellular targets, many anticancer nucleosides need a specific nucleoside transport (NT) proteins in the plasma membranes.¹⁹ The hENT proteins have around 450 amino acid residues²⁰ and another eleven predicted transmembrane domains.²¹ Most human cells contain the plasma membrane hENT1, and hENT1 abundance may be a factor in the response of nucleoside drugs in some cancers.²²

In the study by Kulsoom et al authors detected that bone marrow and peripheral blood hENT1 expression interrelated with each other and with dCK, CDA, dCMPD, and Topo-IIa positively in respective bone marrow and peripheral blood samples. These observations

might indicate a coordinated regulation of expression of these genes suggesting differential transcriptomic regulation.²³

The current study aimed to determine gemcitabine sensitivity in HCC patients treated with gemcitabine. Following previous successful studies, we targeted expression levels of genes responsible for the uptake HENT-1 and metabolism DCK of gemcitabine. By examining gene expression levels in peripheral blood samples from HCC patients, an easy non-invasive method can be used to predict gemcitabine treatment efficacy. No single gene expression level can be used as a predictive marker for gemcitabine sensitivity; however, low levels of HENT-1 expression could be considered as a negative prognostic factor in HCC patients in Egypt. Also, high levels of DCK expression can be assumed as a good predictive marker for gemcitabine sensitivity.

2 | PATIENTS AND METHODS

2.1 | Patients

The study is an analytical cross-sectional study that included 109 consecutive patients consisting of 20 healthy control subjects and 89 patients in whom hepatocellular carcinoma had been identified by liver ultrasonography and/or biopsy and/or computed tomography. The 89 patients were further divided into three groups. The second patient group (HCC control) involved 45 HCC patients who received Se and Vit. E as antioxidants at a dose of 50 and 15 mg/d, respectively, for 4 weeks, while the third patient group (HCC + GEM) consisted of 24 HCC patients who were treated with a combination of gemcitabine (Gemzar, GEM), Se and Vit. E. The dose of gemcitabine was 1000 mg/m² intravenous infusion for 1 week followed by 3 weeks rest. The remaining patient group (HCC + RFA) was consisted of 20 HCC patients who were treated with Se and Vit.E following radiofrequency ablation (RFA) which was performed percutaneously under ultrasound guidance. Our study was implemented according to the 1976 Declaration of Helsinki and its later amendments and approved by our local Ethic Committee. All patients gave their informed consent to the study.

Both healthy control individuals and patients attended Suez Canal University hospital and Communicable Diseases Research and Training Center (CDRTC) in Ismailia city, Egypt, between March 2015 and March 2017. Subjects were excluded if they had a concomitant extra-hepatic tumor, HIV, bronchial asthma, diabetes, or received recent HCC treatment with regimens other than gemcitabine (<6 months) (EASL-EORTC Clinical Practice Guidelines: Management of hepatocellular carcinoma, 2012) (Table 1).

2.2 | Sample collection

From each patient in this study, a sample of 1 mL of peripheral whole blood was collected and stored in a coded sterile EDTA tube. Freshly collected samples were immediately processed for mRNA extraction.

TABLE 1 Groups' names and status

Group name	No. of patients	Status
Healthy control	20	Healthy individuals
HCC control	45	HCC patients with Se and Vit.E
HCC + GEM	24	HCC patients with Se and Vit.E + GEM
HCC + RFA	20	HCC patients with Se and Vit.E + RFA

2.3 | Target mRNA

Expressions of HENT-1 and DCK were examined as genetic predictive markers associated with GEM transport and metabolism, respectively. Specific primers were designed using OLIGO 6.22 software (Molecular Biology Insights, Cascade, CO) (Stam, den Boer et al 2003). The following primers were used for real-time PCR: HENT-1- forward primer, 5'-TGT TTC CAG CCG TGA CT-3' reverse primer, 5'-CAG GCC ACA TGA ATA CAG-3'probe, 5'-(FAM)-CAG CAC CTG GGA ACG TTA CTT-(TAMRA)-3', DCK - forward primer, 5'-TGC AGG GAA GTC AAC ATT-3' reverse primer, 5'-TCC CAC CAT TTT TCT GAG-3'probe, 5'-(FAM)-TAA ACA ATT GTG TGA AGA TTG GGA AG-(TAMRA)-3'.

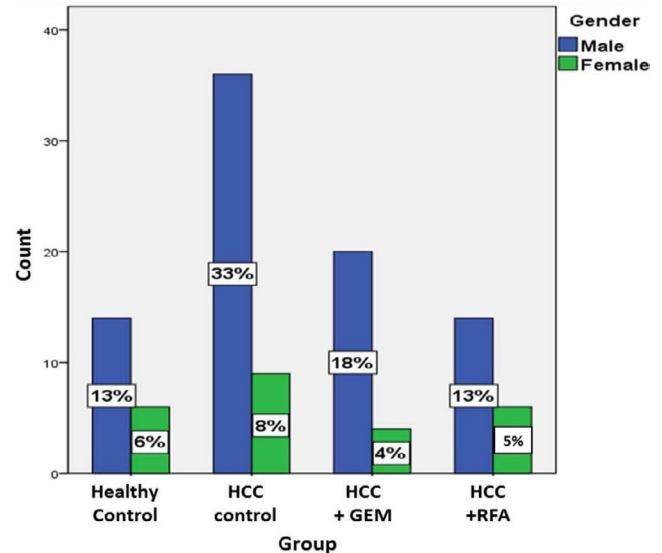
2.4 | mRNA extraction

Extraction of mRNA was performed within few hours of sample collection. Total mRNA was purified from EDTA blood with a fast spin-column procedure (QIAGEN, Hilden, Germany). Red blood cells were selectively lysed and white cells collected by centrifugation and lysed using highly denaturing conditions. After homogenization using the QIAshredder spin column, the samples were applied to the QIAamp spin column and total RNA bonded to the QIAamp membrane and contaminants were washed away, leaving pure RNA to be eluted in 30-100 μ L RNase-free water.

Total mRNA concentration was determined by spectrophotometer, and 1 μ g total RNA was reverse transcribed using a Turbo-1 First-Strand cDNA Synthesis Kit (Biomatik Corporation, Canada). Quantification of the target cDNA and an internal reference gene was conducted by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR).

2.5 | qRT-PCR

Quantitative real-time PCR was performed using the Light Cycler Fast start DNA Master hybridization probes (Roche Diagnostics GmbH), according to the manufacturer's instructions. Each reaction was carried out in a total volume of 20 μ L in glass capillaries, containing 4 μ L of cDNA sample, 25 mmol/L MgCl₂, 10% LightCycler

**FIGURE 1** Gender presentation in study groups

Faststart DNA Master hybridization probes buffer (Taq DNA polymerase, reaction buffer, deoxynucleotide triphosphate mix and LightCycler Fast start enzyme), 10 μ mol/L each primer, and for the probe reactions, 4 pmol (Flu) and 8 pmol (LC).

The cDNA sample was denatured at 95°C for 10 minutes and then added to the capillaries. After this, one cycle of melting curve from 70 to 95°C by a transition rate of 0.2°C/s with continuous detection of fluorescence was performed. The temperature transition rate for all amplifications was 20 °C/s. Negative controls were concomitantly run to confirm that the samples were not cross contaminated. Analysis was carried out with the step one machine (Applied Biosystems).

2.6 | Statistical analysis

Quantitative data will be expressed in the form of mean \pm standard deviation (SD). For differences between more than 2 groups, one-way analysis of variance (ANOVA) was used for parametric variables and Kruskal-Wallis test was used for non-parametric variables to find whether there was a statistically significant difference in between the groups. Qualitative data will be demonstrated through figures of frequency and percentage. Between groups of qualitative data, chi-squared test (χ^2) or Fisher's exact test was used. Data were analyzed using SPSS/PC + 11.5 (SPSS, Inc, Chicago, IL). Statistical significance was set at $P < .05$. Demographic and clinical information was obtained from medical records. Charts of different types will be used to illustrate data and relations where appropriate. To identify relations between different variables, Spearman (ρ) correlation was used. Scatter plot graph was used to illustrate relations between several variables, and to produce a line of best fit and R^2 value of regression.

3 | RESULTS

3.1 | Patient characteristics

Gender distribution of the study population (89 HCC patients and 20 healthy controls) is summarized in (Figure 1) that shows that there was a narrow range of discrepancy in gender distribution and ratio across the study groups.

Totally among the 89 HCC patients, 70 patients were males (78.65%) and 19 were females (21.35%). There was no significant difference between the groups regarding gender.

Regarding age, there was no significant difference between the different studied groups regarding the age.

Concerning etiology, among the 89 HCC patients, 85 (95.5%) patients were HCV-related HCC and only 4 (4.5%) were HBV-related HCC indicating that most HCC cases were mainly due to HCV in Suez Canal area, Egypt. There was no significant difference among different HCC patients' groups ($P > .05$). However, the prevalence of HBV- and HCV-related HCC among HCC patients in the different studied groups was different (Figure 2).

The expression of HENT-1 was significantly increased in the HCC control group relative to the other HCC groups and healthy controls ($P < .0001$). Interestingly, HENT-1 expression was significantly lower in either HCC + GEM or HCC + RFA group ($P < .0001$) as compared to HCC control group. In addition, when compared to healthy control group, HENT-1 expression was not significantly increased in either HCC + GEM or HCC + RFA group ($P = .161$ & $P = 1.000$, respectively) (Figure 3).

Regarding the expression of DCK, it was found to be significantly increased in HCC control group compared to healthy controls ($P < .0001$). Similarly, DCK expression was also significantly increased in either HCC + GEM group or HCC + RFA group when compared to the healthy controls ($P < .0001$). On the other hand, there was no significant difference regarding DCK expression among all HCC groups ($P > .05$) (Figure 4).

4 | DISCUSSION

Worldwide, several biomarkers of gemcitabine sensitivity and effectiveness have been widely studied in multiple types of cancer²⁴⁻²⁶ but less so in hepatocellular carcinoma. However, no molecular biomarkers have been studied in Egypt to predict gemcitabine chemotherapy effectiveness, and patients are empirically treated until patients improve or deteriorate. Therefore, it is tremendously essential to determine predictive molecular biomarkers of gemcitabine resistance and sensitivity for more effective treatment of these cancer patients (Figure 5).

In the present study, we investigated the effect of GEM, RFA, Se, and Vit.E on the expression levels of HENT-1 and DCK in the peripheral blood from different HCC patient groups in Suez Canal University Hospital, and its correlation with clinical characteristics of HCC patients. We found that HENT-1 expression significantly

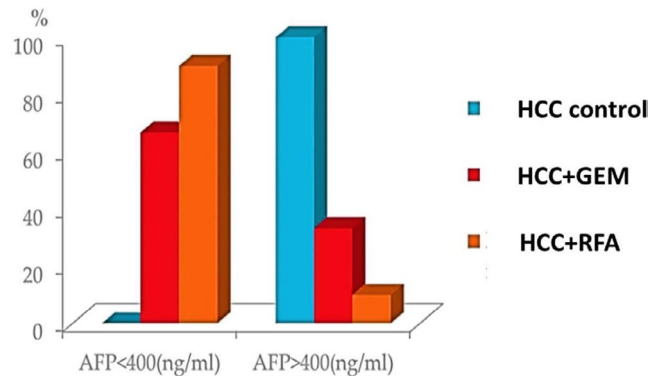


FIGURE 2 Difference in AFP level in study groups (AFP < 400 vs. AFP \geq 400)

increased in HCC control group ($P < .0001$) when compared to the healthy controls and the other treated groups. On the other hand, HENT-1 expression significantly decreased in HCC patients treated with Se and Vit.E in combination with either GEM or RFA when compared to the HCC control group ($P < .0001$). It is worth mentioning that the expression levels of HENT-1 in HCC treated groups (GEM or RFA) were comparable to normal control group levels.

Cellular resistance to gemcitabine can be intrinsic or acquired during gemcitabine treatment.²⁷ Current data came in line with Jiang et al who have reported a high expression of HENT-1 in human hepatocellular carcinoma cell membranes. They explained that the expression of HENT-1 in human hepatocellular carcinoma cells could have a potential clinical value for clinical chemotherapy strategies.²⁸

The HENT-1 protein has been previously estimated as a prognostic biomarker in gemcitabine-treated pancreatic cancer patients as shown by earlier studies^{14,15,29} Elsaleh et al 2009. They reported that higher HENT-1 protein expression levels were related to the increased overall survival and disease-free survival in pancreatic cancer patients under gemcitabine treatment. Also, it was reported by Candelaria²⁴ that downregulation of genes coding for nucleoside transporters and drug metabolism responsible for uptake and metabolic activation of gemcitabine is related to acquired tumor resistance against this agent.

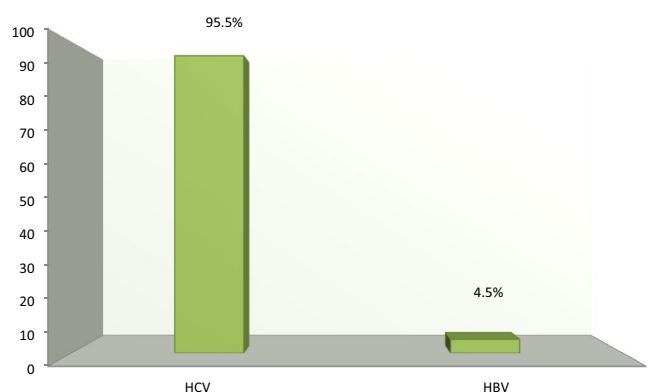


FIGURE 3 The prevalence of HBV- and HCV-related HCC among HCC patients in the study population

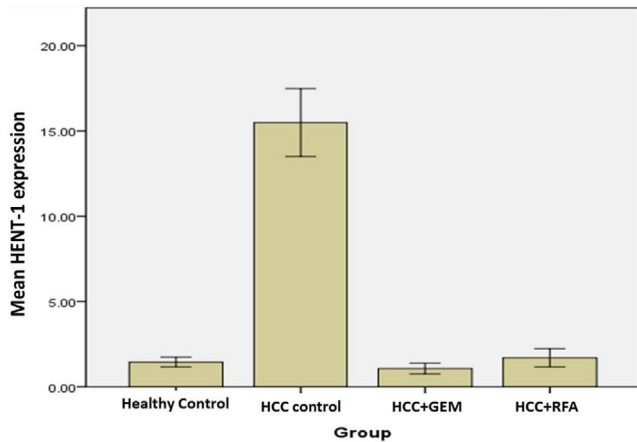


FIGURE 4 HENT-1 expression in HCC patient groups and healthy control

Indeed, the present study revealed that HENT-1 expression decreased after gemcitabine treatment and RFA suggesting decreased drug uptake and bad prognosis. In addition, the RFA-induced reduction in HENT-1 expression currently observed in HCC patients could make them unresponsive to subsequent GEM treatment post exposure to RFA.

Regarding DCK protein expression,³⁰ reported a positive correlation between DCK expression and overall survival, disease-free survival, and reduced mortality in patients with pancreatic ductal adenocarcinoma.³⁰ Moreover, it was reported by Kerl³¹ that decreased DCK expression levels caused gemcitabine resistance in bladder cancer cell lines.³¹ In addition,³² showed that DCK protein expression, using 5 heterotopic transplantation tumor models including several types of malignancies, was closely associated with

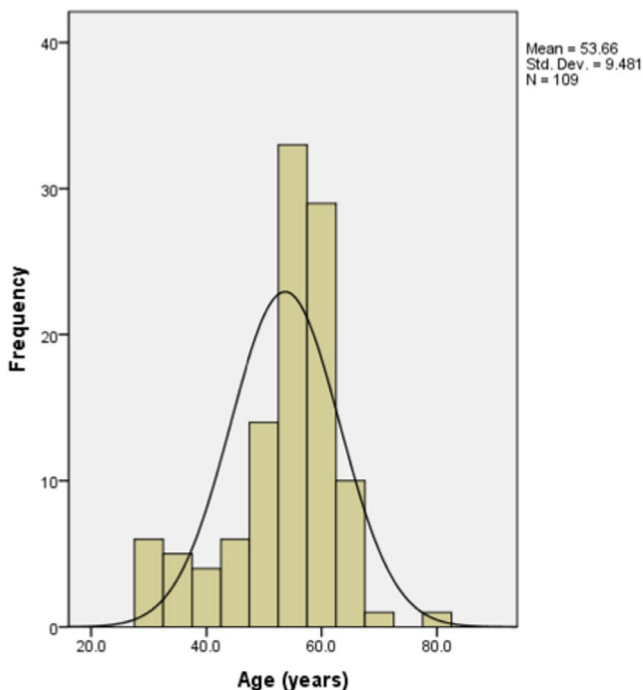


FIGURE 5 DCK expression in HCC groups and healthy control

gemcitabine chemosensitivity. They also proposed that transcriptional regulation in DCK gene and protein expression can be an applicable prognostic marker of gemcitabine response³².

HENT-1 expression is regarded as a possible predictive biomarker for gemcitabine response in some cancers. Gemcitabine is a key chemotherapeutic drug used for the treatment biliary tract cancer (BTC).³³ showed that increased HENT-1 expression is associated with a stronger toxic effect of gemcitabine on BTC cell lines. The study suggested that increased intratumoral HENT-1 is a potential biomarker predicting better therapeutic effects of gemcitabine on advanced BTC patients.^{33,34} proved that downregulating the transcription of hENT1 in pancreatic adenocarcinoma was found to promote gemcitabine resistance.³⁴

High tumoral hENT1 expression on IHC with 10D7G2 is a strong and reproducible prognostic marker for improved outcome among gemcitabine-treated patients with PBC.

Moreover, it was found that 100% of HCC control patients have alpha fetoprotein (AFP) levels greater than 400 ng/mL, while only 33.3% and 10% of HCC patients treated with either GEM or RFA produced AFP greater than or equal to 400 ng/mL ($P < .001$), respectively. This comes in agreement with Patrikidou et al who reported that, in advanced hepatocellular carcinoma (HCC) patients treated with GEM and oxaliplatin, a decrease in AFP level of $\geq 25\%$ was observed in 25% of patients, and a decrease of $\geq 50\%$ was detected in 20% of patients.³⁵ The decrease in AFP level is also indicative of antitumor efficacy, as it is considered one of the most important biomarkers correlated with better overall survival and progression-free survival. Patrikidou and his colleagues suggested that this chemotherapy protocol had stabilized the disease, regardless of the fact it was for a short period.³⁵ From the previous results, we observed that serum levels of the tumor marker AFP were significantly decreased in HCC patients treated with either GEM or RFA compared to HCC controls, suggesting a better prognosis mediated by GEM and RFA treatment.

5 | CONCLUSION

In the current study, we found that DCK expression levels were significantly increased in the HCC control group when compared to healthy controls ($P < .0001$). Paradoxically, either GEM or RFA has maintained the high level of DCK expression detected in HCC control group, suggesting an improved gemcitabine sensitivity and good prognosis. This can be theoretically true, but as DCK is dependent in its activity on HENT-1, increased DCK expression could be of no value in case of decreased HENT-1 expression.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTIONS

Fadia M Attia participated in study design, the sequence alignment and drafting of article, and carried out the laboratory work, carried out the design of the study, and participated by acquisition of data performed the statistical analysis. **Sara Fathy** carried out the analysis and interpretation of data and helped to draft the article. **Mona Elazab** carried out the analysis and interpretation of data and helped to draft the article. **Adel Hassan** carried out the study conception and design, coordination, and drafted the article. **Fawzy Attia** carried out the study conception and design, coordination, and drafted the article. **Gehan Ibrahim** participated in study design and coordination and helped to draft the article. **Maha Anani** participated in study design, the sequence alignment and drafting of article and carried out the laboratory work, carried out the design of the study, and participated by acquisition of data performed the statistical analysis.

ETHICAL APPROVAL

The ethics of this article was approved by the Suez Canal University Ethical Committee.

CONSENT FOR PUBLICATION

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DATA AVAILABILITY STATEMENT

The data can be publically available at the Faculty of Medicine, Suez Canal University.

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