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A Study of Potassium Channel Genes Human Ether a-go-go (heag) and Human ether a-go-go Related Gene (HERG) in Acute Leukemia: Potential Role as a Risk Factor and Impact on Response to Therapy

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ABSTRACT

Background: Human ether a-go-go (heag) and Human ether a-go-go related gene (HERG) are two K+ channel encoded genes, have been related to cancer biology, especially in solid tumors while few studies demonstrate their role in leukemia.

Objectives: Our main aim was to detect the expression of heag mRNA in de novo acute myeloid leukemia (AML) and HERG mRNA in de novo acute leukemia patients, focusing on their prevalence as a risk factor for acute leukemia (AL), their association to FAB subtypes and their response to induction chemotherapy.

Patients and Methods: The study included 84 newly diagnosed acute leukemia patients (64 AML and 20 ALL cases) with 20 healthy individuals as a control group using Reverse Transcriptase-Polymerase Chain Reaction.

Results: The mRNA of HERG was detected in 32/64 (50%) of AML patients and 7/30(35%) of the acute lymphocytic leukemia (ALL) while heag was present in 11/44 (25%) of the AML patients. The expression of both genes were absent in the control group. The highest poor clinical outcome rates were achieved in HERG + patients while the clinical response to the initial chemotherapy was not affected by the presence of heag.

Conclusion: Our findings indicate that HERG and heag genes have an oncogenic potential indicating that they may be necessary for leukemic proliferation. HERG+ is important for risk estimation and prognosis of acute leukemia, while heag gene plays an important role in the pathogenesis of AML.

Key Words: *K+ ion channels – Human ether a go-go gene – Human ether a go-go related gene – Acute leukemia.*

INTRODUCTION

Voltage-gated Potassium K+ channels comprise the largest family of ion channels encoded by ~300 genes with phenotypic diversity. They are not just the main determinants of the cell resting membrane potential, but have also been linked to cell volume control, cell cycle progression, and cardiac repolarization **[1]**.

The ether a-go-go (EAG) family is one of the voltage gated K+ channels that comprises three subfamilies: Human ether a-go-go (heag), Human ether a-go-go related gene (HERG) and ether a-go-go like gene (ELK) **[2]**.

heag channels are expressed exclusively in brain, slightly in placenta and transiently in fusing myoblasts **[3,4]**. The function of heag channels in neurotransmitter release at the neuromuscular junction to initiate action potential in Drosophila melanogaster larvae is well known but their physiological function is unknown in mammals **[5,6]**.

HERG channels are more widely expressed and their functions differ according to their localization. They have a dominant presence in normal human myocardium where being involved in the repolarization phase of the cardiac action potential **[2]**.

In recent years, the role of several voltage gated K+ channels had been described in plethora of malignancies, especially of heag and HERG channel genes. However it is not clear whether these channels play causal role in oncogenesis or whether the oncogenic process result in aberrant expression and activation of EAG families **[7]**.

Several theories have been advanced as how heag and HERG channels could promote malignant transformation. One of these explanations is that cancer cell is more depolarized than normal cell. Accordingly, the overexpression of K+ channels on cell membrane results in hyperpolarization which evokes calcium ions influx resulting in increased transition of cells through G1/S phase of the cell cycle, thereby facilitating cell cycle progression **[8]**.

Another explanation revealed that the presence of hypoxia enables the channels to release hypoxia inducing factor and vascular growth endothelial factor which leads to increased angiogenesis and subsequent invasion and metastasis of tumors **[9]**.

Furthermore, on activation the nuclear localization sequence of the K+ channel results in its perinuclear localization which leads to mitogen activated protein kinase (MAPK) pathway activation resulting in increased cell proliferation **[10]**. Interestingly, the promotor region of HERG harbors multiple binding sites of oncoproteins as the nuclear factor kappa light chain enhancer of activated B cells (NFkB) and there was hypothesis that the mutations of these oncoproteins activate HERG expression **[11,12]**.

The aim of our study was to examine the prevalence of HERG and heag mRNA, focusing on their prevalence as a risk factor for acute leukemia (AL), their association to FAB subtypes and their response to induction chemotherapy.

MATERIAL AND METHODS

Patients:

The study comprised 64 AML and 20 ALL newly diagnosed cases. AML cases included 36 males and 28 females with an age range of 6-75, mean of 40.2±15.6 and median of 41 years while ALL cases included 13 males and 7 females with an age range of 3-54, mean of 19.3±12.2 and median of 17 years. They were provided by both the medical Oncology Department of National Cancer Institute, Cairo University and Beni Suef hospital, Beni Suef University. None of the patients had received any prior treatment and the diagnosis of acute leukemia was confirmed on the basis of complete history taking, clinical examination for hepatosplenomegaly and lymphadenopathy, in addition to the laboratory investigations which included complete blood picture, cytochemical features and immunophenotyping. Informed consent was obtained from all patients involved in the study and from the control group which consisted of twenty healthy individuals with an age range of 5-50, mean of 27.7±13.1 and median of 27.5 years. The patient's characteristics are shown in Table (1).

Methods:

The study of HERG and heag mRNA gene expression was performed by RT-PCR.

1- *Sampling:*

Five ml of venous blood were collected under aseptic conditions in EDTA vacutainer from every patient and control individual.

2- *RNA extraction:*

Total cellular RNA was extracted from the mononuclear cells using the QIA amp RNA blood Mini kit (QIAGEN, Catalogue number 52304) according to the manufacturer's protocol, to be followed by cDNA preparation using ReveRT™ First strand cDNA synthesis kit [Bio Life Scientific products, Catalog#: 5201]. cDNA samples were checked for integrity by PCR detection of human GAPDH using the same conditions for HERG as described below. The primers of GAPDH comprise a sequence between nucleotide 457 to 595 of GAPDH giving rise to a 138bp band. The obtained cDNA was then used as template for the subsequent PCR amplifications of the HERG and heag genes.

3- *PCR reaction:*

Amplifications were performed in a reaction mixture of 3µl cDNA, 12.5µl Master Mix (Fermentas, K 0171 which contains TaqDNA polymerase in reaction buffer, Mg Cl2 and dNTPs), 1µl of 25pmol of each of the forward and the reverse specific primers of HERG and GAPDH, with the addition of water (nuclease-free) to reach a final volume of 25µl in one multiplex PCR reaction assay. The same PCR reaction was used for heag.

The oligonucleotide primers for HERG and GAPDH were as reported by Chernubi et al. **[13]**, HERG primers: Forward 5´ TCC AGC GGC TGT ACT CGG GC 3´; Reverse 5´ TGG ACC AGA AGT GGT CGG AGA ACT C 3´ and GAPDH primers: Forward 5´ AAC AGC

CTC AAG ATC ATC AGC AA; Reverse 5´ CAG TCT GGG TGG CAG TGA T 3´. While the primers used for heag were as reported by Meyer et al. **[14]**, Forward 5´ TCC TCG TTG TAT TTC ACA ATG ACC 3´; Reverse 5´ ATG GGC AAG GGT GGT TTC C 3´.

PCR conditions used for HERG amplification: Thirty-five cycles of amplification were carried out after 2min of enzyme activation at 94ºC; each composed of denaturation at 94ºC; for 30sec, annealing at 56ºC for 1min, and extension at 72ºC for 1min. PCR products were run on a 2% agarose gel stained with ethidium bromide using the 50bp DNA ladder (Fermentas™ Egypt) as a molecular weight marker, and bands were visualized on a UV trans-illuminator. The primers for HERG encompass a nucleotide region from 2171 to 2746 of the HERG cDNA sequence giving rise to 575bp band.

PCR conditions used for heag amplification: An initial enzyme activation step at 94ºC for 3min; 32 cycles of denaturation at 94ºC for 1min, annealing at 59ºC for 1min, and extension at 72ºC for 1min then final extension at 72ºC for 7min. The amplified products were analyzed on 2% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. The positive samples for heag gene were detected at the specific molecular weight of 617bp.

Statistical methods:

Data was analyzed using SPSSwin statistical package version 17 (SPSS Inc., Chicago, IL). Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann-Whitney test. Comparison between 3 groups was done using Kruskal-Wallis test then post-Hoc "Schefe test" was used on rank of the variables for pair-wise comparison. Odds ratio (OR) with it 95% confidence interval (CI) were used for risk estimation. A *p*-value <0.05 was considered significant.

RESULTS

RT-PCR was used to detect mRNA transcripts for HERG K+ channels encoding gene in leukemia cells obtained from 64 AML and 20 ALL patients, meanwhile only 44 out of 64 AML samples were analyzed for the presence of heag expression, as ALL and the remaining AML samples were excluded due to low mRNA content. Both genes expression were examined in 20 healthy control individuals.

HERG mRNA expression was detected in 32 cases (50%), while only 7 cases (35%) of ALL samples expressed HERG; Healthy PB cells did not show detectable HERG expression (Table 2 and Fig. 1).

heag gene m RNA expression was detected in 11/44 (25%) AML cases, significantly higher in patients than control group that did not express heag (*p*–=0.027) (Table 2 and Fig. 2). Patients were identified as HERG+ / heag + or HERG– / heag - according to their expression.

The risk estimate for developing acute leukemia in the presence of HERG mRNA was (Odds ratio 1.44 and 95% CI 1.23-1.70) while the prevalence of heag in AML shows an Odds ratio of 1.6 and 95% CI of 1.3-1.9).

According to the FAB classification, HERG+ expression showed a subtype dependent expression pattern in AML (Table 3). However, the limited availability of samples in each group of FAB subtype was an obstacle for statistical analysis to compare between HERG + and HERG-patients. In ALL, 6/13 (46%) of B-ALL were HERG + patients and 1/7 (14.2%) of T-ALL was positive. heag was positive in 8/20 (40%) M2, 2/7(28.5%) M4, only 1/8 (12.5%) M1.

No association between HERG or heag expression and any of the clinical or laboratory findings parameters was encountered.

The impact of HERG and heag expression on clinical response to the initial chemotherapy

As regards the response of AML patients to induction chemotherapy, the highest remission rate was achieved in HERG– patients, while HERG+ patients had a higher rate of poor response (*p*-value <0.001, odds ratio 33; 95 CI 6.56-165). Similar pattern was shown in ALL (*p*=0.003, odds ratio 4.33; 95 CI 1.60-11.69) (Table 4).

On the contrary, there was no statistically significant difference between patients with heag+ and heag- in AML as regards response to treatment (*p*=1).

Table (1): Clinical and laboratory characteristics of 64 AML and 20 ALL patients.

Patient characteristics	AML patients	ALL patients
Gender:		
Male	36 (56.3%)*	13 (65%)
Female	28 (43.8%)	7 (35%)
Age at diagnosis:		
Years	40.2±15.5 (6-75)**	19.3±12.2 (3-54)
Median	41	17
Blood cell count:		
Hb gm/dl	7.5±1.8 (2.7-11.6)	7.6±2.4 (3.5-11.8)
TLC x 10^9/L	74.9±10.5 (16.9-705)	96.7±90.7 (25-335)
Plt x 10^9/L	50±52.5 (4.0-338)	42.6±24.0 (6.0-96)
BM Blasts %	65±29 (1-99)	80±21.2 (29-99)
Hepatomegaly	43 (67.2%)	13 (65%)
Splenomegaly	26 (40.6%)	12 (60%)
LN enlargement	12 (18%)	16 (80%)
FAB classification:		
M0	3 (4.7%)	
M1	11 (17.2%)	
M2	33 (51.6%)	
M3	6 (9.4%)	
M4	9 (14.1%)	
M6	2 (3.1%)	
T-ALL		7 (35%)
B-ALL		13 (65%)
CR achieved:		
Achieved	24 (37.5%)	10 (50%)
Not achieved	40 (62%)	10 (50%)

Hb : Hemoglobin.
TLC : Total leucocytic count.
Plt : Platelets.
LN : Lymph node.
FAB : French-American-British classification of AML.
CR : Complete remission.
*No(%) **Mean±SD (range).

Table (2): Comparison between AML, ALL and control group as regards HERG and heag expression.

Gene expression	Group			*p*-value
	AML	ALL	Controls	
HERG +ve	32/64* (50%)	7/20 (35%)	0/20 (0%)	<0.001 HS
heag +ve	11/44 (25%)		0/20 (0%)	0.027 S

*No. (%)

Table (3): HERG / heag gene expression in AML in relation to FAB subtype.

FAB type	HERG	heag
M0	2/3 (66.7%)*	0/2 (0%)
M1	4/11 (36.4%)	1/8 (12.5%)
M2	17/33 (51.5%)	8/20 (40%)
M3	1/6 (16.7%)	0/6 (0%)
M4	7/9 (77.8%)	2/7 (28.5%)
M6	1/2 (50%)	0/1 (0%)

*No. (%)

Table (4): The impact of HERG expression achieving complete remission (CR) in AL patients.

Parameter	CR rate		*p*-value
	HERG+	HERG–	
AML	2/32* (63%)	22/32 (68.8)	<0.001
ALL	0/7 (0%)	10/13 (76.9%)	0.003

*No. (%)

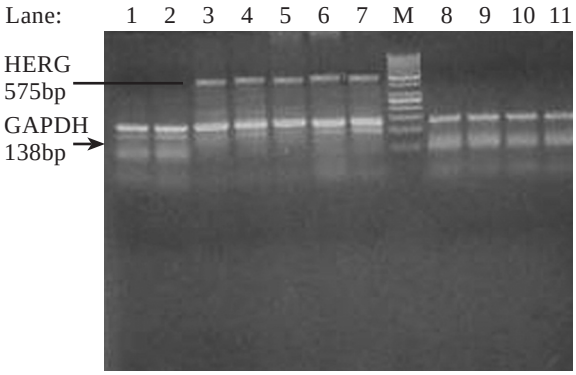

Fig. (1): Electropherogram of HERG expression.
M: 50-1000 bp ladder.
Lane 1 and 2: Negative AML and ALL cases.
Lane 3 to 7: Positive AML cases.
Lane 8 to 11: Control cases.

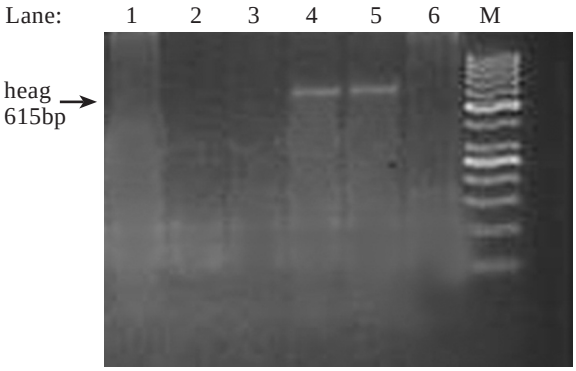

Fig. (2): Electropherogram of heag expression.
M: 50-1000bp ladder
Lane 1-3: Negative AML cases.
Lane 4,5: Positive AML cases.
Lane 6: Control case.

DISCUSSION

Ion channels have been implicated in many diseases, either in a primary etiologic role (Channelopathies) or as mediators in pathogenesis **[15]**. The identification of K+ ion channels and characterization of their functions in tumor cells have stimulated interest in the roles of K+ channels in tumorigenesis and cancer therapy **[16]**.

In the current study, the expression of HERG genes was analyzed in 64 AML and 20 ALL patients. The results showed that HERG expression was detected in both AML, ALL samples but not in normal peripheral blood cells; which this is in accordance with the early previous researches **[17,18,19]**.

The initial study reporting a potential link between the EAG family K+ channels and cancer showed that high HERG mRNA was present in 17 cancer cell lines of different species including human and murine **[20]**. Following this discovery, another group showed that Chinese Hamster ovary cells when transfected with heag gene exhibited a transformed cancer phenotype **[4]**. Many studies showed that HERG gene was overexpressed in many solid tumors **[21-24]**.

It had been reported that HERG K+ channels show differential expression patterns in tumor cell lines. Its overexpression had been suggested to represent a selective advantage for these tumor cells **[25]**.

Two recent studies, provided evidence that HERG mRNA was expressed in almost all the primary leukemia cells, CD34+/ CD38-/ CD123+ leukemic stem cells but not in circulating CD 34+ cells or normal PB mononuclear cells; they reported that HERG expression was not associated with the clinical features of leukemia and its blockage induces growth arrest of leukemia cells in G1 phase which suggests that leukemia cells need HERG channels to proceed with the cell cycle **[26,27]**.

The involvement of heag K+ channels in leukemia has not been systematically studied, although its relevance in many solid tumors is well established **[28]**. Smith et al. **[19]** did not detect heag in chronic lymphocytic leukemia, while Agarwal et al. **[29]** did not detect it in ALL; therefore, authors concluded that heag is not relevant in lymphatic leukemia **[29]**.

In the current study, we examined heag expression in 44 AML patients; heag gene was detected in 11/44 (25%) of AML and none of the control cases. The positive cases included 8/20 (40%) M2, 2/7 (28.5%) M4 and 1/8 (12.5%) case was M1. Using REAL time PCR, Agarwal et al. **[29]** detected heag expression in 47/118 (40%) of AML cases. The expression has showed a subtype dependent pattern; M2 and M4 were the most common subtypes that expressed heag, This is similar to our study taking in consideration that M2 and M4 were the most frequent subtypes analyzed.

The current study showed that the prevalence of HERG or heag may play an important role as a risk estimate factor in leukemia. Our study also showed that HERG+ expression in acute leukemia patients had adverse response to the initial induction chemotherapy which reflects poor prognosis.

The prognostic value of HERG expression in tumor cells has been evaluated in several tissues. Studies showed that HERG K+ channels were involved in different aspects of tumor establishment, progression and mediating tumor invasion **[30,31]**. In AML patients, HERG+ expression is associated with a higher probability of relapse, shorter time to relapse and shorter overall survival time compared with HERG- AML patients **[32]**.

It was hypothesized that HERG K+ channels regulated different aspects of acute leukemia pathophysiology such as survival and proliferation in the bone marrow **[24]**, cell motility and transendothelial migration possibly through PIK/AKT **[32]** or stromal cell-derived factor-1 (SDF-1/CXCR4) pathway **[33]**.

In the current study, HERG+ ALL cases included 6/13 (46%) B-ALL and 1/7 (14.2%) T-ALL. In a recent study, Pillozzi et al. **[34]** examined both HERG m RNA and protein expression in ALL by using real time PCR and Flowcytometry, both methods detected overexpression of HERG transcript in ALL blasts, also, it was shown that HERG expression correlated with that of CD10+ precursor B-ALL. Authors reported that leukemic cells are known to be protected from chemotherapy by mesenchymal stem cells (MSC) and HERG channel function appears to be important for this effect. Thus, agents that exclusively target HERG may

be adequate to overcome MSC-induced drug resistance **[34]**.

heag could be used as an additional prognostic factor in AML as it significantly correlates with a bad prognosis **[29]**, this was in contrast to our study as heag positivity in AML cases did not affect their initial response to chemotherapy, but this finding would require the support of prospective studies in a large sample population.

Blocking heag and HERG channels inhibits cell proliferation and disease progression, but since both heag and HERG belong to the same family of K+ channels and shares 47% of the amino acid sequence, both channels are affected by the same drugs. The use of different drugs to block these genes as monoclonal antibodies **[35]** and small interfering RNA **[36]** are expected to provide such an advantage in cardiac cases. The same might apply to acute leukemia.

In conclusion, both HERG and heag have an oncogenic potential, by affecting the proliferation of leukemia cells which suggests that both K+ channels are molecular markers for human neoplastic haematopoietic cells. HERG+ is important for risk estimation and prognosis of AL, while heag gene has more important role in leukomogenesis of AML rather than its prognosis which needs more justification by a large prospective study. Both K+ channels are considered as a potential pharmacological targets for cancer therapy.

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