



***n-ras* and *k-ras* Mutations Among Some Iraqi Patients with Acute Lymphoblastic Leukemia**

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Abstract

Somatic mutations which influence the activity of RAS genes, *n-ras*, *h-ras*, and *k-ras*, are recognized mainly in codons 12, 13, and 61 of exons 2 and 3 in human cancers. This persuades to search in coding genes suspected to be contributing to leukemogenesis such as *n-ras* and *k-ras* mutations that may associate with acute lymphoblastic leukemia for some Iraqi patients using a combination of high resolution melting-polymerase chain reaction (HRM-PCR) scanning with sequence analysis. This study was carried out on fifty Iraqi patients (34 male, 16 female), (2-70 years of age) with acute lymphoblastic leukemia (ALL), including 20 pre-treated, 15 under-treated and 15 relapsed patients compared with a sample consisted of 50 apparently healthy individuals as control group collected randomly from population living in Baghdad (4 - 66 years of age).

Of 50 ALL Iraqi patients, 6 patients were identified with an abnormal melting curve showing low frequency of *ras* genes mutations, this result is translated a poor prognostic for *ras* mutations in ALL. According to the results of this study the c.34 in exon 2 (codon 12) was a hot spot for *n-ras* mutations and c.35 in exon 2 (codon 12) was a hot spot for *k-ras* mutations in ALL Iraqi patients, while no codon 61 mutations were detected for either *n-ras* or *k-ras* gene.

Keywords : acute lymphoblastic leukemia, *n-ras* gene, *k-ras* gene, mutations, HRM-PCR

Introduction

Ras is an important cellular switch, relaying growth promoting signals from the plasma membrane to the nucleus (Lintig *et al.*, 2000). There are three closely related cellular RAS genes, encoding the highly homologous N-, H-, and K Ras 21-kDa proteins. All three RAS genes show activating mutations in human tumors (Fernández-Medarde and Santos, 2011; Prior *et al.*, 2012). Somatic, gain-of-function mutations in *ras* genes were the first specific genetic alterations identified in human cancer. Thus, Fernández-Medarde and Santos (2011) have characterized the Ras proteins as

essential components of signaling networks controlling cellular proliferation, differentiation, or survival. The oncogenic mutations of the *h-ras*, *n-ras*, or *k-ras* genes frequently found in human tumors are known to throw off balance the normal outcome of those signaling pathways, thus leading to tumor development. It is believed that *ras* mutations drive the proliferation of leukemic cells (Liang *et al.*, 2006). Mutations in *n-ras* and *k-ras* have long been recognized as a recurring molecular event in childhood acute lymphoblastic leukemia (cALL), with a reported incidence of between

15% and 22% (Tartaglia *et al.*, 2004 ; Wiemels *et al.*, 2005) . The objective of this study was to investigate the association of *n-ras* and *k-ras* mutations with acute lymphoblastic leukemia (ALL) in some Iraqi patients.

METHODS

Samples

The study is carried out on fifty ALL Iraqi patients (34 Male, 16 Female) aged 2-70 years ,including 20 pre-treated , 15 under-treated and 15 relapsed . The clinical diagnosis of the patients had been made at a consultant medical staff in the following medical centers : The National Centre of Hematology / Al Mustansyria University ; Central Pediatric Hospital and Baghdad Teaching Hospital. Fifty apparently healthy individuals aged (3-75 years) had been selected to be matched with the patients according to age, gender and ethnicity (Iraqi, Arabs), they are non-smokers and without indication of any exposure to therapeutic or diagnostic irradiation or other potential genotoxic substances for one year prior to blood collection. No member of either group had consumed alcohol (wine and/or beer). Before beginning the study, all participants are informed about the objectives and experimental details of this research and to complete a standardized questionnaire to obtain necessary data on life styles and personal factors (age, occupation, health, etc). Blood samples from all cases and control had been subjected to the mutation analysis investigations.

Blood samples from all cases and control had been subjected to DNA

extraction , HRM-PCR and sequencing to detect mutations at codons 12, 13, and 61 of the *n-ras* and *k-ras* genes.

DNA extraction

Genomic DNA was extracted from blood samples of all cases and control group using Qiagen Mini kit (Qiagen Ltd.) . Amplified by PCR, and the amplicons were subsequently screened for mutations.

DNA extraction was performed according to the manufacturer instructions . After DNA extraction, agarose gel electrophoresis is adopted to confirm the presence and integrity of the extracted DNA molecules using a UV transilluminator . Electrophoresis of DNA is only to ensure the success of DNA extraction for each sample before starting PCR.

High Resolution Melting Curve - PCR (HRM-PCR)

Genomic DNA samples of ALL patients are analyzed by high resolution melting (HRM) PCR to determine mutations in *n-ras* (12 , 13 , 61) and *k-ras* (12,13 ,61). For each high resolution melting (HRM) analysis, a Roche light cycler 480 along with Light cycler 480 high resolution melting Master kit (Roche Diagnostics co./ Germany) was used , this was conducted in the ultra-clean reagents preparation room.

Primers

Primer sets are designed through primer 3 plus software primer sequences to cover the mutation hotspots in *n-ras* and *k-ras* genes. Primers that are used for HRM-PCR and sequencing reactions are available in table 1

Table 1 : A list of the primers that are used for HRM-PCR and sequencing reactions .The primers sequences below is written 5' to 3'.

Name of	Sequence of primers 5'→ 3'
N-RAS 1213-	GAAAGCTTTAAAGTACTGTAGATGTGG
N-RAS 1213-	AGGTCAGCGGGCTACCACT

Name of	Sequence of primers 5' → 3'
N-RAS-12	GAAAACTACTCCAGAAGTGTGAGG
N-RAS-12	GGATTGTCAGTGCGCTTTTCCCAACACCACGTG
N-RAS-12	TGGTGGTGGTTGGAGCACGTGGTGGTGGGAAAAGC
N-RAS-12 CM-	CAGAACTGTTAAAAACTGGACAGG
N-RAS-12 CM-	GAAAACTACTCCAGAAGTGTGAGG
N-RAS-12	GGATTGTCAGTGCGCTTTTCCCAACACCAGTTG
N-RAS-12	TGGTGGTGGTTGGAGCAAGTGGTGGTGGGAAAAGC
N-RAS-12 CM-	CAGAACTGTTAAAAACTGGACAGG
N-RAS-12 CM-	GAAAACTACTCCAGAAGTGTGAGG
N-RAS-12	GGATTGTCAGTGCGCTTTTCCCAACACCAGCTG
N-RAS-12	TGGTGGTGGTTGGAGCAGGTGGTGGTGGGAAAAGC
N-RAS-12 CM-	CAGAACTGTTAAAAACTGGACAGG
N-RAS-12 CM-	GAAAACTACTCCAGAAGTGTGAGG
N-RAS-12	GGATTGTCAGTGCGCTTTTCCCAACACCAACTG
N-RAS-12	TGGTGGTGGTTGGAGCAGTTGGTGGTGGGAAAAGC
N-RAS-12 CM-	CAGAACTGTTAAAAACTGGACAGG
N-RAS- 61-F	CCCCCTTACCCTCCACAC
N-RAS- 61-R	TATCCGCAAATGACTTGCTA
KRAS 1213-F	TTATAAGGCCTGCTGAAAATGACTGAA
KRAS 1213-R	GGTTTCTCTGACCATTTTCATGA
KRAS 61 -F	TTTTTGAAGTAAAAGGTGCACTG
KRAS 61 -R	GACCAGTACATGAGGACTGGG

All required primers are thawed completely and vortexed for at least 15 sec.

HRM-PCR analysis is performed in the presence of the cyto 9 dye (Applied Biosystems ,USA) and LightCycler 480 (Roche Diagnostics, Germany). For each sample the reaction mixtures with a final volume of 20 µl had been made off with the reagents listed in Table 2 .

Table 2 : The reagents that are used for HRM-PCR and the amount required per melting curve reaction of one sample.

Materials	Concentration (µl) / sample
TBE Buffer	2.0
MgCl ₂ (25mM)	1.28
dNTPs	0.35
Enzyme	0.25
Water	10.5
Syto 9 HRM dye	1.0
Primer Mix	0.62
DNA	4.0
Total reaction volume	20

The PCR reaction mixes are prepared for each amplicon , as per the volumes in the HRM template . 16 µl of the reaction mixes is aliquoted in the assigned wells according to the HRM template .Wild-type (WT) DNA is used as negative controls throughout the study .4 µl of DNA is added to the relevant well on the plate according to the template layout starting with the negative controls (N1/N2) .The wells are sealed except for the positive control ones.

HRM Amplification

The HRM plate is taken to the HRM-PCR equipment laboratory and placed in the designated hood. 4µl of positive controls is added to the relevant wells. The wells are sealed and centrifuged for 3 min. After that, the plate is taken to the LC480 light cycler .Then , the light cycler (LC480) and the computer are switched on and programmed as shown in table 3, the fluorescent level is continuously recorded.

Table 3: HRM-PCR program

PCR Program		
Temp °C /Time		Cycles
96°C/1min		15
96°C/10 sec		
84 °C/1.02 min		
96°C/10 sec		5
60°C/1min		
96°C/10 sec		5
64°C/2.05 min		
4°C		Store
HRM Program		
Stage	Temp/Time	cycles
1	95°C/10min	
2	95°C/15 sec	10
	67°C/1min	
3	95°C/15 sec	5
	62.5°C/1min	
4	95°C/15 sec	35
	71°C/3min	
	85.5°C/20sec	
	62.5°C/1min	
5	95°C/15 sec	
	50°C/1min	
	95°C/15 sec	

The change of fluorescence is converted to a melting peak (melt curve) by plotting the negative derivative of the fluorescent signal corresponding to the temperature on the light cycler software. Light cycler 480 gene scanning software (HRM V2.0 mutation surveyor software) (Applied Biosystems ,USA) is used to aid

mutation identification. Mutations are identified by the comparison of each patient's peak plot with that of W.T. and mutant reference DNA.

HRM Analysis

HRM analysis is conducted for gene scanning after the completion of the run of HRM-PCR. The curve for the negative and positive controls was observed to notice clear differences. The samples that were analyzed were observed to determine if they appear as wild-type or mutant. Samples that appeared as mutant or possible mutant (i.e. with no wild-type patterns) directly sequenced after purification.

Purification

Following HRM-PCR and prior to sequencing, the PCR products are purified, using the Charge Switch PCR clean-up kit (Life Technologies / UK) according to the manufacturer's instructions.

Sample Electrophoresis

After purification, the specificity of all primer sets are checked by running the amplicons from the high resolution melting analysis on an agarose gel, i.e., The HRM-PCR products were separated, using gel electrophoresis to estimate the quality of the product. Thus, the resultant PCR products from HRM-PCR were electrophoresed in 2% agarose and visualised on a gel electrophoresis system to determine amplification of the target exons.

Dye Terminator Cycle Sequencing of the PCR Products

The sequencing reaction mix is shown in table 4.

Table 4: The reagents for the sequencing and the amounts necessary.

Reagents	Required per sample(μl)
2.5 Big Dye Terminator	0.7
5X Sequencing Buffer	2
Primer (1pmol/μl) (F or R)	1
Purified PCR Product	3
H ₂ O	3.3
Total	10

After determination of an aberrant HRM profile and purification of a PCR product, the purified PCR product is used as template in cycle sequencing to confirm the presence of a mutation and determine its position in the exon, using the ABI big dye terminator ready reaction kit v3.1 (Applied Biosystems, USA) (table 4). For each exon sequencing reaction (without the DNA template) mixtures were made. The mixes were placed for vortexes to spin briefly in microfuge. Finally they were stored on ice until use, and then the remainder is discarded.

An appropriate number of strips are labeled so that the samples can be easily identified. Each sample has a forward and reverse reaction for each exon. The reactions are run on a Verti 96 well thermal cycler (Applied Biosystems, USA) according to Sequence DT-55 cycle-sequencing cycling program as shown in table 5.

Table 5: Sequencing reaction program

Temperature (°C)	Time (min/sec)	Cycles
96	1min	1
96	10s	15
60	1min 20s	
96	10 s	5
60	1min 35s	
96	10s	5
60	2min 5s	

Clean up of Sequencing Products

After cycling , the product is proceed to the next purification .Clean up of sequencing products was performed using a gencourt cleanSEQ kit (Beckman Coulter ,USA) , according to the manufacturer's instructions .

Sequencing

Sanger sequencing (Seth *et al.* , 2009 a, b) is usually regarded as the 'gold standard' for mutation detection. After purification PCR product is analyzed on an Applied Biosystems 377A automated DNA sequencer (Applied Biosystems , USA), following the manufacturer's protocol (Applied Biosystems , USA).

Results and Discussion

As many articles describe the detection of point mutations with the LightCycler (LC) technology (Loeffler *et al.* ,2000 ; Lay *et al.* ,2006) and as it is now known that the majority of ALL cases are characterized by distinct genetic alterations and sequence mutations , low frequency of *ras* genes mutations were observed within 50 ALL Iraqi patients , where only 6 patients (12%) showing *ras* gene mutations translating a poor prognostic for *ras* mutations in ALL .While, another study on the same 50 ALL Iraqi patients suggests that *notch1* gene is a good prognostic for ALL Iraqi

patients (Al Amili *et al.* ,2014) . This relatively low incidence of *ras* mutations in ALL has suggested that *ras* plays a minor role in the pathogenesis of this leukemia.

Of those 6 *ras* mutated patients , 3 patients were with *n-ras* mutations (2 were with *n-ras* G12C and one was with *n-ras* G12S) and 2 were with *k-ras* mutations (one were with *k-ras* G12V and one was with *k-ras* G12A), while one ALL patient have a mutation in *n-ras* and *k-ras* gene in the same time (G12C for *n-ras* mutation and G12V for *k-ras* muatation) (Table 6) . These findings are in line with the preferential mutation pattern of the *ras* gene observed by others (Neri *et al.* ,1988 ; Yokota *et al.* ,1998 ; Nakao *et al.* , 2000 ; Tyner *et al.* , 2009 and Mizutani *et al.* , 2012). Interestingly, no codon 61 mutations are detected for either *n-ras* or *k-ras* gene. Mutational activation of Ras via a point mutation at codon 12, 13 or 61 is well characterized as a marker for progression of normal or benign cells toward malignancy (Parikh *et al.* , 2007; Parikh and Ren, 2008). These distinctions for different mutations may be clinically important as some preclinical data suggest that different mutations might activate different pathways. Whatever the explanation for the differences in mutations, it

appears that the function of all these detected mutations in *n-ras* and *k-ras* are important in ALL cells or that any of the potentially different biological effects can contribute to ALL development. Table 6 shows that c.34 in exon 2 (codon 12) was a hot spot for *n-ras* mutations and c.35 in exon 2 (codon 12) was a hot spot for *k-ras*

mutations in ALL Iraqi patients according to our results.

Activating mutations in the Ras oncogenes are among the most common genetic alterations in human cancers, including patients with ALL (Perentesis *et al.*, 2004). However, it is believed that Ras mutations drive the proliferation of leukemic cells (Liang *et al.*, 2006).

Table 6 : Analysis of N-Ras and K-Ras mutations.

c. : Nucleotide Position ; G* : Glycine ; S : Serine ; C : Cysteine ; V : Valine ;

Patient code	age group (	Group of ALL Patient	Ras gene	Exon (codon)	Nucleotide position	Nucleotide change	Predicted a.a. change	Type of mutation
Pt.39	11-20	Under-treated	N-Ras	2 (12)	c.34	Substitution GGT>AGT	G* 12 S	Missense
Pt.10	21-30	Relaps-ed	N-Ras	2 (12)	c.34	Substitution GGT>TGT	G* 12 C	Missense
Pt.23	11-20	Pre-treated	N-Ras	2 (12)	c.34	Substitution GGT>TGT	G* 12 C	Missense
Pt.17	31-40	Relapsed	N-Ras	2 (12)	c.34	Substitution GGT>TGT	G* 12 C	Missense
			K-Ras	2 (12)	c.35	Substitution GGT>GTT	G* 12 V	Missense
Pt.1	11-20	Pre-treated	K-Ras	2 (12)	c.35	Substitution GGT>GTT	G* 12 V	Missense
Pt.9	11-20	Relaps-ed	K-Ras	2 (12)	c.35	Substitution GGT>GCT	G* 12 A	Missense

A: Alanine .

Of those six ALL patients , three were at relaps stage ,two were at pre-treated stage ,and one was under –treated. All mutations were at exon 2 codon 12 and all were missense . All *ras* mutations were missense point mutations occur at codons 12 , 13 and 61 (Bowen *et al.* , 2005 ; Mascaux *et al.* , 2005).Unfortunately , among three *k-ras* mutation hot spots, *k-ras* codon 12 mutation induces higher resistance to apoptosis and predisposition to anchorage-independent growth than codon 13 mutations (Guerrero *et al.*, 2000). A point mutation of the *n-ras* gene is one of the known genetic alterations identified in patients with

acute lymphoblastic leukemia (ALL), but its clinical importance is controversial (Yokota *et al.*, 1998) .

As some studies report that a high prevalence of *k-ras* mutations is found in pancreatic (56%), colorectal (51%), uterine (15%), and ovarian (11%) cancers (De Rooock *et al.*, 2010 and Forbes *et al.*, 2011), Zhang *et al.* (2009) suggested that oncogenic *k-ras* mutation initiates leukemia in hematopoietic stem cells.

Four of six *ras* mutated ALL Iraqi patients were at 11-20 years age . Janku *et al.* (2011) propose that *n-ras* mutation status is not significantly associated with age, gender, or race.

The presence of *n-ras* mutation in children with ALL may be an independent predictor for worse clinical outcome and therefore may have therapeutic implications (Lübbert *et al.*, 1990) . In this study ,the clinical relevance of *n-ras* and *k-ras* gene mutations in ALL patients remains controversial , partly because rather small series of patients have been analyzed. Thus, examination of *ras* in further prospective trials comprising many more (larg-scal) patients could determine the prognostic significance of this type of oncogene activation in ALL.

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