H. M. Al-Khafaji
Applied Science Department
University of Technology-Baghdad-Iraq
truthy_2006@yahoo.com

Gene Expression Bcl-2 Gene in Cancer of Brest in Iraqi population

Abstract *The breast cancer is dangerous disease in the world . Molecular methods are important and necessary to diagnose breast cancer. Many of the genes with expression change like Bcl-2 gene is specifically, coding an anti-apoptotic protein and and therefore classified as an oncogene. Determine the damage of Bcl- 2 gene as a cause of some types of cancer, such as breast cancer, leukemia, prostate cancer and lung cancer .In this study, we examined Bcl-2 expression levels in (malignant, benign and healthy) tissues of the breast .They were fifty Laboratory samples (18 cancer tumor , 12 benign and 20 marginal (non-cancer) breast tissue that diagnosed based on their information were obtained from their files and records in all patients in this study , to extract the DNA and measure the level of expression of gene under study by molecular technique of (r –t PCR). Expression of gene under study is higher levels in malignant group and the fold of expression was 10.00 time higher than the control group and also in the benign group the fold of expression was 2.18 that higher than the control group.The results showed the expressed gene Bcl-2 is significantly higher in the third grade of breast tumor samples with Ct (22.14) of the first grade with CT (25.63) and the second with CT (24.07). According to the results of the study that the use of molecular methods in measuring the expression of Bcl-2 gene may help to diagnose the disease and may be considered that the Bcl2 gene is molecular tool for the early detection of breast cancer*

Received on: 28/9/2016
Accepted on: 23/2/2017

Keywords- *Breast Cancer - gene expression - real-time PCR*

How to cite this article: H. M. Al-Khafaji "Gene Expression Bcl-2 Gene in Cancer of Brest in Iraqi population " *Engineering and Technology Journal*, Vol. 35, Part B, No.2, pp. 119-125, 2017.

1. Introduction

Cancer starts when normal cells in breast alteration and grow uncontrollably, formation mass called a tumor. A tumor is classified into two categories: malignant, (cell grow and spread to other parts or organs in the body), and Benign (tumor will not spread) .With more than one million new cases in the world each year (1) the breast cancer is important malignancy and comprises 19% of all women cancers. Breast cancer incidence rates are rising in the world, mostly in the developing nations. In 2013, an evaluated 232,350 women were expected to be diagnosed in the US with breast cancer, with 39,630 women were expected to die (2) *BCL2* gene is located on Chromosome 18and its size:196,783 bases **Figure(1)** and encode Bcl-2 (B-cell' lymphoma, 2) protein in human being, is the one of members of the Bcl-2 family regulatory proteins that organized the death of the cell (s) either by triggering (pro-apoptotic) or prevent (anti-apoptotic) apoptosis.(3) (4) . Gene Bcl-2 is encoded a anti-apoptotic protein seriously and therefore classified as an oncogene, Identified the damage of the Bcl-2 gene may be reason for a number of cancers, including breast and prostate cancer, leukemia, lung cancer (8) (17) Malignant tumor may be diagnosed and its degree of severity not only from cell proliferation but also from the rate of cell death.(5) (6)

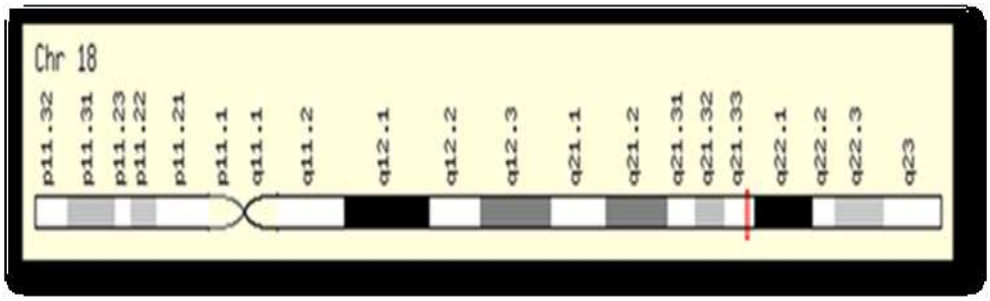
Bcl-2 gene expression may be considered an apoptosis markers in breast cancer tissues. The majar purpose of this research is to discover the expression of Bcl-2 in breast tumor tissue and compared the results with marginal samples as a control. (7)

2. Materials and Methods

I. Samples

For design of this study, 30 tissue samples from patients, were obtained of the biopsy or following, a surgical mastectomy.,All samples were Collected from Al-Amel National Hospital for radiation\ Baghdad. Samples were divided in to groups including:- first:18 malignant tumors second: 12 benign breast tissues and third: 20 non tumors (marginal) as a control which was all pathologically diagnosed(8)

https://doi.org/10.30684/etj.2017.138623
2412-0758/University of Technology-Iraq, Baghdad, Iraq



Figure(1): *BCL2* gene

II. DNA extraction

Total DNA of all samples was extracted using the ReliaPrep Blood gDNA Miniprep System. Promega_USA according to the protocol provided by the manufacturer . Expression of Bcl-2 was measured by (qRT–PCR) (9) (10) (11) The gene expression levels and fold change were quantified by measuring the threshold cycle (Ct) employing the KAPA SYBR® FAST qPCR Kits components. Every reaction was done in a duplicate.

The primers of Bcl-2 were synthesized by Alpha DNA Ltd (Canada) , matched with the NCBI, stored lyophilized at (-20°C). The following primers for Bcl-2 were included in the reactions:

Forword Sequence(5'->3'):
GAGTGACAGTGGATTGCAT
Reverse Sequence(5'->3'):
CAGAATATCAGCCACCTCTT

GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) housekeeping gene was used as an internal control to be used in calculating the □CT value.

The primers of GAPDH were synthesized by Alpha DNA Ltd (Canada stored lyophilized at (-20°C). The following primers for GAPDH were included in the reactions:

Forword Sequence (5'->3'):
CTATAAATTGAGCCCGCAGCC
Reverse Sequence (5'->3') :
ACCAAATCCGTTGACTCCGA

Each 20 µl of the qRT–PCR reactions contained 2 µl, DNA (100 ng), 10 µl KAPA SYBR® FAST qPCR Master Mix, 6.4µl RNase free water, 0.6 µl of each primer (300nM) , and 0.4 µl of Rox/Low were used.

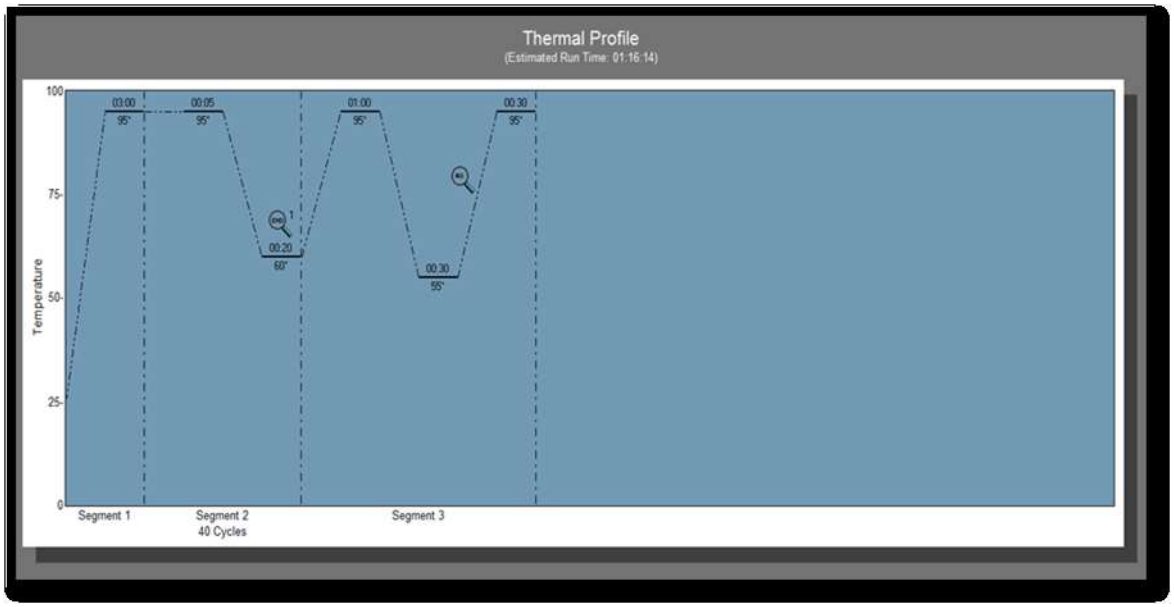
The thermal profile of gene in this study expression rt PCR is shown in Table (1).The qPCR Reaction run was programmed according to the thermal profile.

III. Statistical analysis :

The means and standard deviations were recorded for each sample (test and control) variables included Ct values and gene expression levels .

Table (1): Thermal profile of Bcl-2expression

Step	Temperature	Duration	Cycles
Enzyme activation	95ºC	30 sec	Hold
Denature	95ºC	5sec	40
Anneal/extend	62ºC	20 sec	
Dissociation	1min /95 ºC-30 sec /55 ºC-30sec/95 ºC		



Figure(1) :Thermal profile used in expression of *Bcl-2*.The profile was taken directly from qPCR machine

3. Results

I. Results of Total DNA Extraction

Total DNA was successfully extracted from all samples .The concentration of total DNA ranged from 60 to 160 ng/ µl.A high concentration with yield a of total DNA depends on the extraction conditions with strict aseptic techniques .

II. Results of primer optimization of GAPDH and bcl2 genes (concentration and annealing temperature).

The optimum concentration of *GAPDH* foreword and reverse primers were 300 nM and 300 nM. The optimum concentration of forward and reverse primers for *bcl2* were also 300 nM and 300 nM as shown in Table (2) optimization of primer concentration is an important step in any PCR reaction.

Table (2): Concentration and Annealing Temperature of *GAPDH* and *bcl2* genes

	GAPDH	bcl2
Forward primer concentration	300 nM,	300 nM
Reverse primer concentration	300 nM	300 nM
Annealing temperature	59 ºC	60 ºC

III. Results of Q(Real Time PCR):

Quantification rt PCR applied in this experiment utilized SYBR green(a fluorescent dye) which recognizes any double stranded DNA including cDNA. (8)

IV. Real time PCR quantification of GAPDH Expression

The Ct value of *GAPDH,* the housekeeping gene used in the this study is shown in Table (3) Range of Ct value for *GAPDH* in the patient with malignant tumor was (25.34-26.10) and for benign tumor it ranged from (25.29-26.01). The variation of total change in expression of *GAPDH* was studied in two study groups utilizing the 2^{-Ct} value as shown in Table (3).Figure(2).

The 2^{-Ct} value of benign tumor group was3.79 E- 8 Malignant tumor group, it was 3.42E-8 and the 2^{-Ct} value for control group was4.04 E- 8.

The computed ratio for gene fold expression was1for the control groups , 0.93 for the benign tumor group and 0.93 for the Malignant tumor group

Table (3): Comparison of GAPDH Fold expression between study groups

groups	Means Ct of *GAPDH*	2^{-Ct}	experimental group/ Control group	Fold of gene expression
Malignant tumor group	24.80	3.42E- 8	3.42E- 8/ 4.04 E- 8	0.84
benign tumor	24.65	3.79 E- 8	3.79 E- 8/ 4.04 E- 8	0.93
Control	24.56	4.04 E- 8	4.04 E- 8/ 4.04 E- 8	1.00

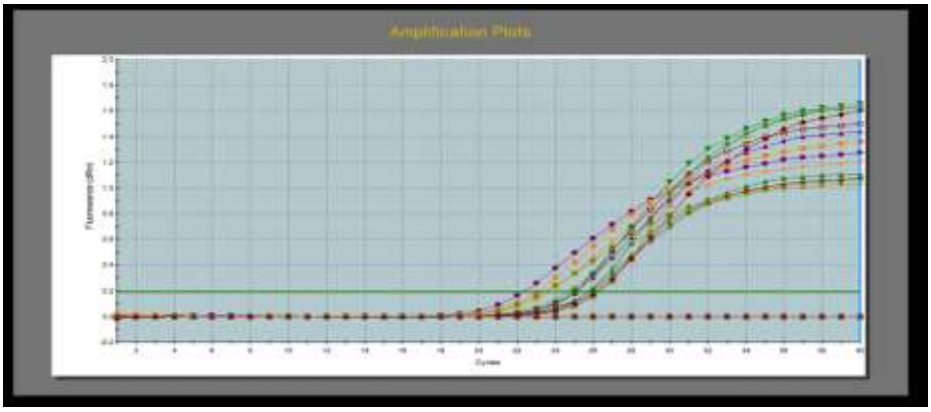


Figure (2) *Bcl2* amplification plots by qPCR. Samples included all study groups .Ct values ranged from 22 to 24 .

Table (4). Comparison between different groups in Ct, ΔCt and $2^{-\Delta Ct}$ value (*Bcl2*) (Mean ± SD)

Group	No	Mean ± SD of Ct	Range	Mean ± SD ΔCt	Range	Mean ± SD $2^{-\Delta Ct}$
Malignant tumor group	18	27.86± 1.77	25.04-28.45	4.95±2.2	1.46-8	0.15±0.18
benign tumor group	12	30.03±0.81	28.62-32.01	6.12±1.6	2.89-8.14	0.02± 0.03
control	20	31.01±1.01	29.93-33.31	6.99±1.9	3.43-9.08	0.01±0.02

V. Real time PCR quantification of bcl2 Expression

The mean±SD Ct value of bcl2 DNA amplification was (23.86± 1.77) in Malignant tumor group. It ranged from 20.04 to 27.45.The range of Ct values in benign group was from 28.62 to 32.01with a mean ± SD (30.03±0.81) and the range of Ct values in control group was from 29.93 to 33.31 with a mean ± SD (31.01±1.01) . Table (4).

The mean Ct values of control group were higher than the malignant tumor group. This is important in reflecting the original mRNAs present in the samples

.

It is It is evident from these results that the malignant tumor group is associated with the highest copy number of mRNAs reflecting its higher expression

Each quantificative PCR reactions was run in triplicate for each sample, In each run , samples from malignant group and control group were run in addition to non template and non- primer controls. This was important to make the statistical calculation of each group and in order to specify the calibrator .Plots of each run were recorded including the amplification plots and dissociation curves.

Figures (3) (4) show the amplification plots and dissociation curves for *bcl2* .

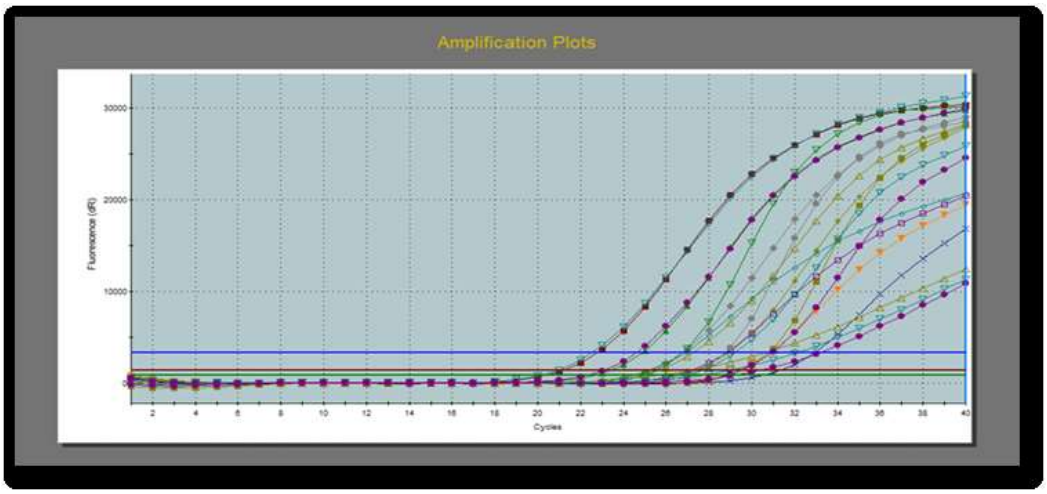


Figure (3) *Bcl2* amplification plots by qPCR. Samples included all study groups .Ct values ranged from 22 to 30 .

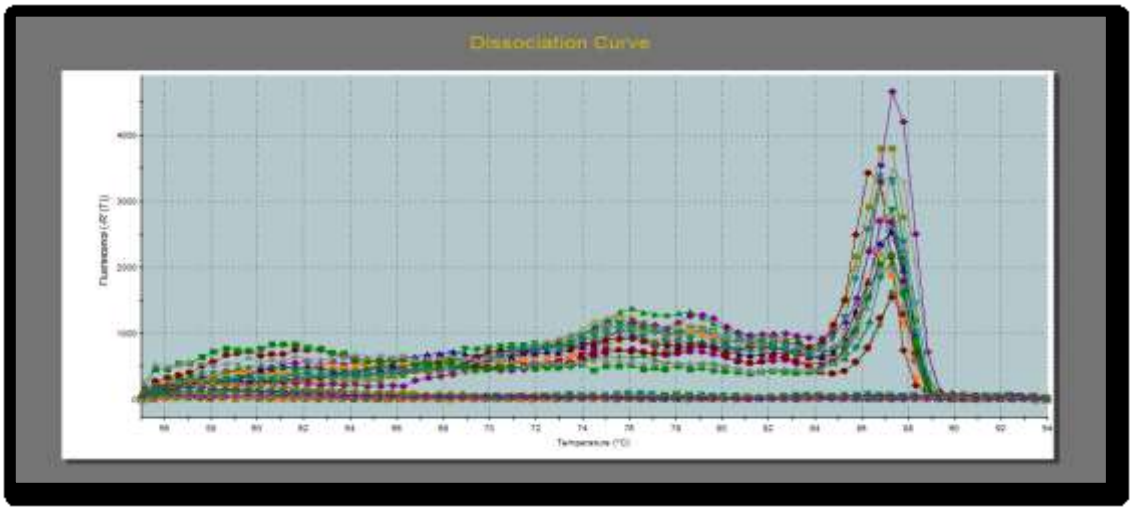


Figure (4) *bcl2* dissociaition curves by qPCR.Samples included all study groups. Melting temperature ranged from 85°C to 87°C, No primer dimer could be seen.

Table (5). Fold of *XRCC1* expression Depending on $2^{-\Delta Ct}$ Method

groups	Means Ct of *bcl2*	Means Ct of *GAPDH*	ΔCt(Means Ct of *bcl2*- Means Ct of *GAPDH*)	$2^{-\Delta Ct}$	experimental group/ Control group	Fold of gene expression
Malignant group	27.86	24.80	3.06	0.11	0.11/0.011	10.00
Benign group	30.03	24.65	5.38	0.024	0.024/0.011	2.18
control	31.01	24.56	6.45	0.011	0.011/0.011	1.00

Table (6): comparison between grade of tumors

Grade	No. of samples	Mean ± SD of Ct	Range
I	5	25.63±1.1	24.11-27.45
II	7	24.07± 0.66	23.32-24.99
III	6	22.14 ±1.5	20.04-24.16

VI.Normalization of Ct (cycle threshold) Values

In the present study, quantitative RT -PCR assay analyzed the mRNA expression of Bcl2 and compared its expression between benign tumor group as control group and malignant group. The calculation of gene expression fold change was made using relative quantification. (12)

This depends on normalization of Ct values calculating the ΔCt which is the difference: between the mean Ct values of replica of bcl2 DNA amplification of each single case and that of the GAPDH.

Results are shown in Table (5).The fold of gene expression in mlgninat group was 10.00 time higher of the control group and the fold expression of benign group was 2.18 that higher than the control group. These results indicate increase expression of bcl2 gene in malignant and benign groups.

VII. Expression Bcl-2 gene and degree of tumors:-

In this study, according to the results, the gene expression of Bcl2 was associated **Table (6)**: comparison between grade of tumors

VIII. Quantitative rt PCR

Molecular molecular technique r –t PCR quantification applied in the present experiment utilized the SYBR green ,a fluorescent dye which recognizes any double stranded DNA including cDNA , The amplification was recorded as a Ct value (cycle threshold). The lower Ct value indicates the presence of higher copies of the target and vice versa. In terms of gene expression, high values of Ct, indicate low, expression of gene and low value of Ct indicates a high gene expression (12) (13)

IX. Real time PCR quantification of GAPDH Expression.

The variation of total change in expression of GAPDH was studied in study groups utilizing the 2-Ct value The results show small variations in gene fold expression between the study groups renders GAPDH gene a useful control gene.The benefit of use the housekeeping genes in this study is that their expression remains constant in tissue under investigation (14).This agrees with Robert et al., 2005. They studied the expression of 1,718 genes using qRT-PCR they applied the GAPDH as a indicator gene in 92 types of normal human tissue .and found that using of GAPDH is good method for the normalization in qRT-PCR when applied in clinical studies(15).

X. Real time PCR quantification of bcl2 expression.

The mean of Ct values was different between the study groups .The mean Ct values of control group were higher than the malignant and benign groups. This is important in reflecting the original mRNAs present in the samples .It is evident from these results that the patients group is associated with the highest copy number of mRNAs reflecting its higher expression . bcl2 gene encodes an integral outer mitochondrial membrane protein blocks the apoptotic death of some cells Bcl-2 proteins contribute to programmed cell death or apoptosis that explains this difference. Bcl-2 may be a prognostic biomarker in breast cancer (16)(17) .

XI. Expression Bcl-2 gene and grades of tumors -:

Compare between of degree of tumors and expression of Bcl-2 gene showed that high gene expression was related to the degree of advanced tumor . Tang et al. (2013) also showed a good link between Bcl-2 expression and metastasis to the nodes of lymph .

In another study which was immunohistochemically analysed the expression of the Bcl-2 protein in 223 female breast cancer, They found that the Bcl-2 expression were inversely related to tumor grade, tumor necrosis, oestrogen and progesterone receptor content, However heterogeneous expression of the Bcl-2 protein was associated with high grade . Early detection, of breast, cancer plays the important role in reducing mortality rates.

References

[1].S. L. Blachford. "The Gale Encyclopedia of Genetic Disorders.". Detroit Gale Group, Thomson learning. Vol.1. p: 1345. (2002.

[2].American Cancer Society, (2013).

[3].C. J. Moldoveanu and M. T. Llambi , "The Bcl-2 family reunion." Molecular Cell, 37, 299-310. (2010).

[4].C. P. E., Lessene, G. A.Strasser, and J. M. Adams, "Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy." Nat. Rev. Mol. Cell Biol. 15, 49–63 (2014).

[5].L.M. Merlo , J.W. Pepper, B.J .Reid and C.C. Maley "Cancer as an evolutionary and ecological process". Nature Reviews Cancer. 6 (12): 924–35. (2006).

[6].D.A.Shamas and D. W. Andrews, "Mechanisms of action of Bcl-2 family proteins.

"Cold Spring Harb. Perspect. Biol. 5, a008714 (2013).
[7].J.M. Hardwick and L.B. Soane "Multiple functions of BCL-2 family proteins". Cold Spring Harb Perspect Biol. 5 (2): a008722. (2013).
[8].S.M. Dawson,S. N. Makretsov and F.M. Blows "Bcl-2 in breast cancer: a favourable prognostic marker across molecular subtypes and independent of adjuvant therapy " J Cancer, 103, 668-75. (2010). [9].S.C. Bustin, V.V. Benes and E. T. Nolan "Quantitative real-time RT-PCR—a perspective.". J Mol Endocrinol. ;34:597–601.(2005).
[10]S.S. Taylor, Wakem, M.T. Dijkman and G. M. Alsarraj, "A practical approach to RT-qPCR-Publishing data that conform to the MIQE guidelines". Methods. 50 (4): S1–5. (2010).
[11].J.X. Ye., Coulouris G.K. , Zaretskaya and I.Z., Cutcutache "Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction". BMC Bioinformatics, 13: 134. (2012).
[12].K.J. Livak,. and T.D. Schmittgen, "Analyzing real-time PCR data by the comparative CT method". Nature Protocols 3, 1101-1108. (2008).
[13].T. R. Nolan and E.S. Hands "Bustin Quantification of mRNA using real-time RT-PCR" Nat Protoc, 1 (3), pp. 1559–1582. (2006).
[14].E.A. Reboucas, J.T. Costa, and M.Y. Passos. "Real Time PCR and Importance of Housekeepings Genes for Normalization and Quantification of mRNA Expression in Different Tissues". BRAZILIAN ARCHIVES OF BIOLOGY AND TECHNOLOGY;56: 143-154. (2013).
[15].B.T. Robert , W.T. Harmer and A.W. Coleman " GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues". Physiol Genomics.; 21: 389–395. (2005).
[16].P. E. Rongqing,and L. J. Hogdal. "Selective BCL-2 inhibition by ABT-199 causes on-target cell death in acute myeloid leukemia". Cancer Discovery. 4 (3): 362–375. (2014).
[17].A. R. Delbridge, and A.Strasser, "The BCL-2 protein family, BH3-mimetics and cancer therapy". Cell Death Differ. 22, 1071–1080(2015).

Hiba Muneer AL- Khafaji, Ph.D. in Genetic Engineering , Institute of Genetic Engineering and biotechnology \ university of Baghdad . Her main areas of research interest are genetic engineering and cancer genes. Dr. AL- Khafaji is currently lecturer in Applied science Department, biotechnology branch University of Technology, Baghdad, Iraq.