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Detection of Deletion Mutation in Exon 2 of the NRF2 Gene and its Association with Glutathione Level in Breast Cancer

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Abstract **Original Research Article**

NFE2 related factor 2 (NRF2) is a major mechanism in the cellular defense against oxidative or electrophilic stress. The activation of the Nrf2 is needed in the production of antioxidant elements such as Glutathione. So any defect in the NRF2 gene may affect the production of antioxidant molecules. The aim of the current setting is the detection of a deletion mutation in exon 2 of the NRF2 and its association with the level of glutathione in Breast cancer with late stages. The current study was conducted on Sudanese females with Breast Cancer who attended the Institute of Nuclear Medicine – Madani – SUDAN between August to October / 2018. The procedure was included in the serological part to measure Glutathione in plasma and molecular part to detect deletion mutation in the NRF2 gene (exon 2). We found a higher level of glutathione in patients compared with the control p-value < 0.0001. In addition to that, we obtained a positive correlation between a high level of glutathione and different breast cancer stages (r = 0.882), also a deletion mutation in NRF2 exon 2 in 15% of patients and 3% in control was found. Our findings suggest that it could be there is a relationship between increased level of glutathione and progressive late stages in patients but this increased it may not attribute only to exon 2 deletion mutation of the NRF2, maybe there are unknown factors are participate in this increasing.

Keywords: Glutathione; Breast Cancer; NRF2; Antioxidant.

ABBREVIATION

NRF2	NFE2-related factor 2
Keap1	Kelch ECH-associated protein 1
EDTA	Ethylenediaminetetraacetic acid
GSH	Reduced Glutathione
ARE	Antioxidant Response Element
XRE	xenobiotic response element
PCR	Polymerase chain reaction
DDW	Double Distal water
TAE	TAE (Tris-acetate-EDTA)
ROS	Reactive Oxygen Species
SPSS	Statistical Package of Social Science
RT-PCR	Reverse transcription polymerase chain reaction
NGS	Next-Generation Sequencing

INTRODUCTION

NFE2-related factor 2 is considered as a major mechanism in the cellular defense against oxidative or electrophilic stress because it is responsible for the production of antioxidants molecules [1, 2]. These molecules are produced to detoxify and eliminate the reactive oxygen species (ROS) [3]. The production of antioxidant molecules are controlled by multiple genes, but the NRF2 gene is the most important gene in controlling antioxidant. So any mutation in this gene leads to an abnormal level of antioxidant.

The upregulation of the NRF2 in certain cancer cells creates a suitable environment for cell growth and protection against anticancer therapeutic drugs [4]. Although the NRF2 has advantages in the early stages of tumorigenesis when the host is looking for controlling premalignant carcinogenesis, it also has disadvantages in

later stages when could make fully malignant cancer cells become resistant to treatment [5-7]. That means the dark side of the Nrf2 represents the resistance of chemotherapeutic agents [8-10]. However, the regulatory mechanisms involved in mediating Nrf2 activation are not fully understood [11].

NFE2L2 gene located on chromosome 2, has 6 exons, approximately 34.8 kb in size, contains two promoter regions ARE / EpRE (antioxidant or electrophile response element) and regulatory region contains three XRE (xenobiotic response element) 12, 13]. There are many types of mutations in NRF2 exon 2 that have been demonstrated by previous studies [14, 15]. Some of these studies have analyzed the splice variants in oncogenes showed that such tumors express abnormal transcript variants from the encoding Nrf2 gene that lack exon 2, or exons 2 and 3, and encode Nrf2 protein which leads to disrupt the interaction between NRF2 and KEAP1 (Kelch ECH-associated protein1) domains [16]. The deletion in this exon responsible for the increased level of antioxidants, when the mutation occurs at any coding region of the NRF2 that may enhance the production of antioxidant molecules (such as glutathione) which facilitated the escaping of cancer cells from killing by chemotherapeutic agents [17, 18]. Glutathione (GSH) is a ubiquitous intracellular peptide and considered as master antioxidant molecules inside the body [19-21]. Regarding the relationship between cancer and Glutathione, elevated levels of Glutathione in the tumor could protect cancer cells [22-25].

The aim of recent study to detect the presence or absence of deletion mutation in exon 2 of the NRFE2 gene and its correlation with the level of glutathione (GSH) in a Breast cancer patient with late stages, because thought to be there is a relationship between mutant NRF2 gene and level of Glutathione in progressive stages.

MATERIAL AND METHOD

Study Design

The cross-sectional, (descriptive observation study) was conducted on the female with Breast Cancer patients with late stages who attended the Institute of Nuclear Medicine – Al Gezira state – SUDAN between (August to October - 2018). The population was divided into 100 (patients) and 100 (control). Data were collected using self-administered questionnaires.

The study was approved by Ethical and Scientific Committee of the College of Medical Laboratory Science, al- Gezira University and Ethical Committee from Ministry of health – al- Gezira State as well as permission from statistic Department in the Institute of Nuclear Medicine, Molecular Biology and Oncology -Madani. Informed consent was obtained from all subjects after their agreement to participate in this study. In addition to, we confirmed that all experiments were performed in this study in accordance with relevant guidelines and regulations.

The study population was divided into 50 % patients and 50% control see Figure-1 for distribution.

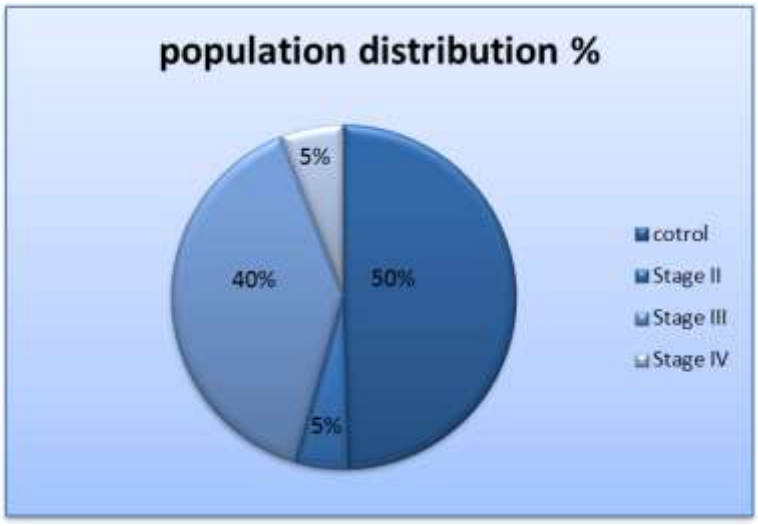

Fig-1: Showing distribution of population

The procedure was divided into two parts:

Part one – serological part

Whole EDTA Blood samples were collected and centrifuged at 3000 rpm/10 mints. Plasma was separated and kept in Eppendorf tubes at -20 ºC for a couple of weeks later. To measure reduced glutathione the quantiChrom Glutathio assy kit (DIGT-250) was used [26]. The kits are composed of two reagents; reagent A: 30 ml, reagent B: 30 ml, and calibrator: 10 ml (equivalent to 100µM. First, all reagents were equilibrated to Room Temperature. 120µL of the sample (plasma) mixed with 120 µL of reagent A in 1.5 ml centrifuge tubes mixed and vortex well. 200 µL of the mixture (sample + reagent A) was transferred into 96

well plates and 100 µL of reagent B was added. Tap the plate lightly to mix. Incubated at room temperature for 25 min. Read at Optical Density 412 nm.

Part two – molecular part

Apparatus which used in this part were included heating block, Convenient PCR, Electrophoresis, and Gel-documentation system.

To extract DNA, GENEKAM DNA ISOLATION KIT (Ref. SB0071-74) was used. The kit is composed of 4 solutions: solution A (sodium hydroxide), solution B (Buffer), solution C (solution), and solution Z.

Solution (Z) was Prepared freshly before used (Note: follow the manufacture instruction of how to prepare solution Z).

Procedure

25 µL of whole EDTA blood was added to 100 µL freshly prepared solution Z in a 1.5 ml tube and kept at 88 **°C** for 7 minutes in the heating block. 100 µL of solution **B** was added to 1.5 ml tube, then immediately vortex for 10 seconds. 200 µL of solution C was added. Finally, the tube stored overnight at 4 **°C** and the supernatant used as a source of target DNA.

PCR Protocol

DNA 5 µL (DNA concentration was diluted to 100 ng), primer 5µL (R/F Con 30 nM), PCR Master mix 12.5 µL (Master mix was purchased from INTRON BIOTECHNOLOGY OG-180905-108) and complete the volume up to 25 µL by (DDW).

The sequence of PCR primer was designed used primer plus 3 programs.

Primer	**(sequences 5′→3′)**
Forward primer:	5′TTG ACA TAC TTT GGA GGC AAG A3'
Reverse primer:	5'TTC TGA CTG GAT GTG CTG GG 3'

PCR Program

PCR was programmed as following: Denaturation 94 **°C** for 2 mint, annealing 55 **°C** for 1 mint, Extension 72 for 2 mint, cycle 30 cycles and final Extension 5 mint.

Electrophoresis Protocol

2g of agarose powder was mixed in 100 mL 1xTAE (Tris-acetate-EDTA) in a microwavable flask and left for 2 mints in the microwave until Agarose completely dissolved. The Agarose solution was let to cool down. 3 µl of Ethidium Bromide was added to the solution then the Agarose solution was poured into a gel tray with the well comb in place (poured slowly to avoid air bubbles). The tray was let at room temperature 20 mints until completely solidified. The tray was filled with buffer 1xTAE until covered the gel. Carefully the samples (PCR bands) were loaded into wells. Besides samples, the DNA ladder (size 100 bp) was used to measure the length of the bands. To visualize the electrophoresis bands, a gel documentation system was used.

RESULT

Data were statistically analyzed using the SPSS program version 21. Statistics used were the mean, standard deviation (SD), Confident Interval (CI) 0.05, Pearson correlation (r), and P-value. P-Value of <0.05 was considered significant and > 0.05 was considered insignificant.

- All values are expressed in Mean ±SD.
- The age of subjects (patients and control) were range between 39- 55 years.

For the level of Glutathione in different stages see Table-1.

Table-1: Level of reduced Glutathione in different stages

stages	**Level of Reduce glutathione (µM)**
Stage II	48.19 ±12.77
Stage III	109.14 ±22.35
Stage IV	182.00 ±27.82

For the level of Glutathione in population see Table-2.

Table-2: The level of reduced Glutathione in population

Parameters	**Patients (mean± sd)**	**Control (Mean ±sd)**
Level of reduced Glutathione (µM)	113.11 ± 20.98	13.87 ±9.27

For the correlation between plasma GSH levels and Breast Cancer stages see Chart-1.

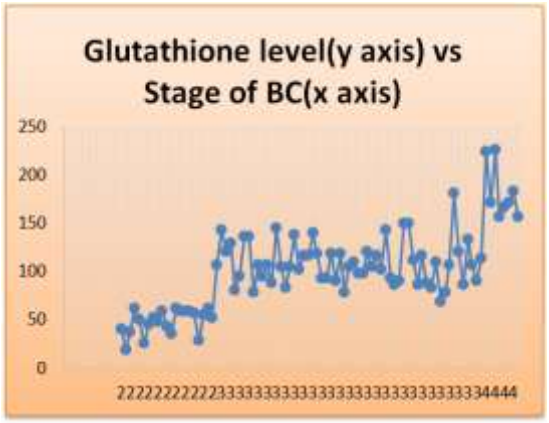

Chart-1: Glutathione (y axis) vs. Breast Cancer Stages (x axis)

Chart-1 illustrates, there was a positive correlation (r = 0.882; p- value < 0.0001) between stages and high level of the glutathione in patients.

For the DNA purity and concentration in population see Table-3**.**

Table-3: DNA parameters in population

DNA parameters	Patients	Control
Purity	1.95 ± 0.08	1.81 ± 0.35
Concentration (ng)	200.7 ± 69.24	201.10 ±54.37

For the Agaros gel electrophoresis of control see Figure-2 and for patients see Figure-3.

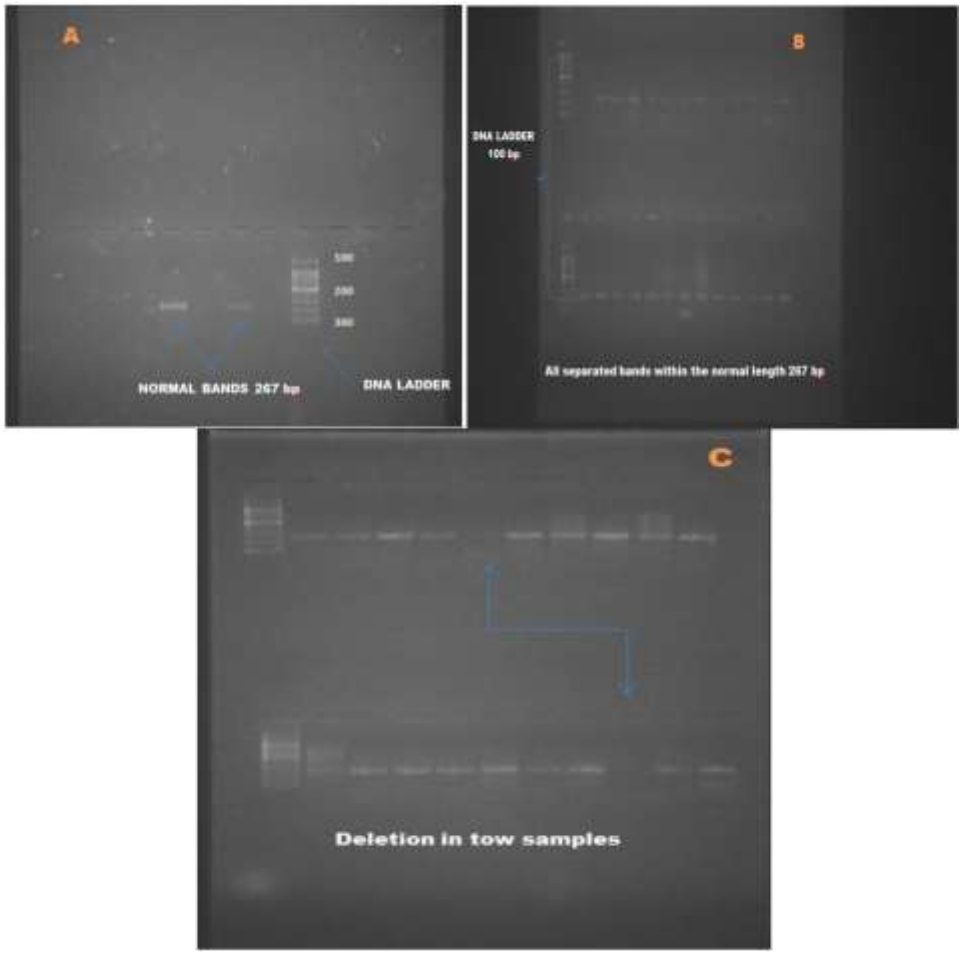

Fig-2: Agarose gel electrophoresis shows the result of control. DNA ladder (100 bp) was used to compare target bands with ladder. (A) And (B) No deletion, all samples within the normal length of the band (227 bp), (C) only deletion appeared in 2 samples

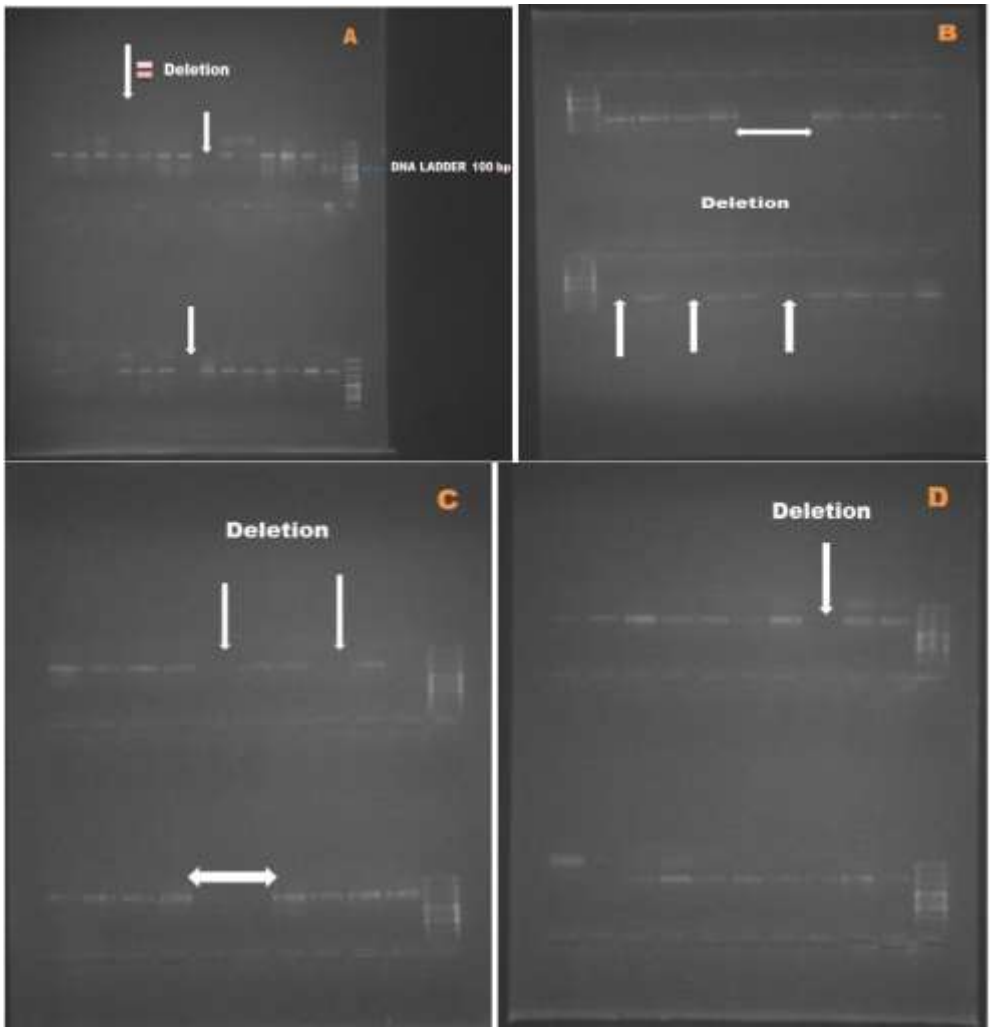

Fig-3: Agarose gel electrophoresis shows the result of patients. DNA ladder (100 bp) was used to compare target bands with ladder. (A) deletion in 2 patients , (B) deletion in 5 patients , (C) deletion in 3 patients and (D) deletion in 1 patient

For the distribution of mutation (%) among the population see Table-4.

Table-4: The distribution of absent / present of mutation among the population

population	Absent of exon 2 %	Present of exon 2 %
patients	**15%**	**75%**
Control	**3%**	**97%**

DISCUSSION

Accumulating evidences have demonstrated that the mutations of the NRF2, have a critical role in the activation of NRF2 [27]. Most of the mutations in NRF2 are somatic mutations [28, 29].

The common known mutation in NRF2 is the loss of the exon 2 gene. The mutant NRF2 gene couldn't bind with KEAP1, without properly binding between NRF2 and KEAP1, the proteasomal degradation of the NRF2 doesn't occur [30, 31]. Thereby, resulting in a persistent localization of the NRF2 in the nucleus. Due to the accumulation of the NRF2 in the nucleus, occurs a transcriptional activation of the NRF2 target genes to produce antioxidant molecules [32-34]. One of the most important antioxidants molecules produced is Glutathione. So this could be a direct relationship between the NRF2 mutant gene and the level of glutathione. The results showed that the level of Glutathione in different stages was significantly higher in patients (p-value < 0.0001) when compared with control. We obtained also a positive correlation between a high level of glutathione and different stages (r = 0.882), so maybe there is an association between increased level of glutathione in patients and progressive late stages. This is in agreement with the results of earlier studies which have demonstrated that an increased level of glutathione in Breast Cancer Patients [35, 36]. As well as for NRF2 exon 2 we found a deletion mutation in 15% of patients and that is in agreement with previous studies that have demonstrated the presence of deletion mutation in exon 2 of the NRF2 gene [37, 38]. However, 75 % of

patients appeared a normal exon but their glutathione level was high. That indicates cancer cells may use other mechanisms to enhance their Glutathione level.

The other mechanisms may include another type of mutations in other exons. Nevertheless, to know exactly which types of other mutations present, we need to do sequencing (Sanger or NGS), and that was not involved in our recent study but it could be done later with further studies.

So we could not assert that increased level of Glutathione in patients is attributed only to defect (deletion mutation) in exon 2 of the NRF2 gene, especially 3% of control has a deletion mutation, however, they have a normal glutathione level. In addition, some patients even without deletion mutations, but have a high level of Glutathione. To figure out more about the causes which lead to elevate the Glutathione level in Breast Cancer Patients and its role in late stages we need long term follow-up of patients. Nevertheless, for accuracy and for scientific credibility, the validity of the recent results couldn't be approved until the additional experiments such as RT-PCR and western blots should be included.

CONCLUSION

Our findings suggest that it could be there is a relationship between the high level of Glutathione and progressive late stages in patients but this increased perhaps not attribute only to exon 2 deletion mutation of the NRF2, maybe there are unknown factors are participated in this increasing. Further studies on a large sample size are necessary to confirm the findings of this study.

Author Contributions

Nagia Suliman: Conceptualization, investigation, methodology, visualization, statistical analysis, and writing–original draft; Omer Balla: formal analysis, Supervision, and review.

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