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RELATION BETWEEN CYTIDINE DEAMINASE ACTIVITY AND BREAST CANCER

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Abstract

Cytidine Deaminase (CDA) is an important gene which encodes an enzyme that regulates the pyrimidine salvage pathway. It catalyzes the hydrolytic deamination of cytidine to products such as uridine and ammonia. The expression and activity of CDA is influenced by different treatments given to breast cancer patients. Aim of this study is to evaluate the expression analysis of CDA along with its protein activity on the basis of substrate utility. RNA was extracted from the blood samples of 35 cancer patients which comprised of 26 breast cancer patients and 9 samples from other cancer patients. 10 healthy controls were taken for comparison. Integrity of extracted RNA was validated through cDNA synthesis which was then utilized in qRT PCR to check the expression levels of CDA. Enzymatic activity was analyzed by testing ammonia levels released during enzymatic reaction of CDA. Our study reveals that CDA expression and activity increases in breast cancer patients as compared to other cancer patients and the control group. CDA expression was found to be elevated in radiotherapy patients and CDA activity was increased in patients receiving hormone therapy. These molecular insights into gene expression and enzymatic activity of CDA can provide clinical guidance in future for tailoring effective treatment strategies. Our findings suggests that the increased expression and activity levels of CDA can worsen the effects of breast cancer so therapeutic approach can be adopted in this regard for the silencing of CDA gene expression and lowering its enzymatic activity in breast cancer patients by introducing specific novel drugs.

INTRODUCTION

Breast cancer is one of the most common kinds of cancer that has affected many women globally. Therefore, this disease has become a challenging field of interest for scientists. As far as surgery is concerned, which has ruled the area of cancer therapy has greatly caused significant disfigurement when the surgical instrument is applied to the organ such as breast. The studies regarding history of BC are complex to understand because of the molecular pathways involved in it. The most common type of therapy adopted by doctors in past was the physical removal of tumor by surgery or targeted hormone therapy to the cell receptors. Moreover, it remains a common field of interest to find the gadgets responsible for early detection of breast cancer (Lakhtakia, 2014). It is the second most common cancer which causes death

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among women worldwide. It has been observed that about 42000 women die of BC every year, that makes it fifth leading cause of death in the world. In 2020, about 2.3 million women were affected with BC and about 685,000 deaths are caused by it annually. (Ben-Dror *et al.*, 2022).

It is quite surprising that all women despite of their ethical or racial heritage are at risk of breast cancer at some stage of their life. BC has been detected 23% among the women worldwide. WHO reports more than 1.2 million women suffer through this disease annually. Moreover, it has been revealed in another study that one in 9 women in Pakistan is diagnosed with breast cancer which ranks as one of the most common diseases in Asia. According to the reports stated by Shaukat Khanum Memorial Hospital, the incidents rate of BC is about 46% in Pakistani women (Asif *et al.*, 2014). It has been reported at surgical unit from Civil Hospital Karachi that Israel is the top Asian country which has highest number of breast cancer patients compared to Pakistan which comes second in this regard (Memon *et al.*, 2015).

Mastectomy involves the removal of the whole of the breast part. The term has been derived from the Greek word Mastos, which means woman's breast and a Latin word ectomia, which stands for excision. The radical mastectomy is a kind of procedure in which the entire breast part gets eliminated including lymph nodes, muscles (Goethals & Rose, 2023).

In 1971, The National Surgical Adjuvant devised a random study to resolve dispute over surgical management of BC. The results indicated that there is a slight difference between survival rate of women who were treated with Halsted surgery and those who were dealt with less extensive surgery (Fisher *et al.*, 2002)

The invention of hormonal therapies in the mid-20th century, such as tamoxifen, revolutionized the treatment of breast cancer. These drugs work by blocking the production of estrogen, a hormone that can stimulate the progression of some types of breast cancer. For the past quarter century, tamoxifen has been declared as a basic standard for the treatment of estrogen receptor positive BC. About 400,000 women are surviving today because of this drug therapy and millions have taken benefit from it. Keeping in view its significance, World Health Organization (WHO) has listed tamoxifen as a necessary drug for the treatment of BC (Jordan, 2003). It has also been shown in a study that Peiminine stimulates the arrest of G1/G0 inducing apoptosis and autophagy as depicted in the Figure 1. (Yu *et al.*, 2021). The advancements in imaging technologies such as ultrasound and mammography, have improved the early diagnosis of BC. Mammography reduces the mortality rate resulting from BC by detecting tumors at an earlier stage. The United States Preventive Services and Task force analyzed results from random trials of mammographic screening. The results revealed that the decrease in mortality due to screening was 22% in women of age 50 years or older, and 15% in the women of age 40-49 years. Overall, history of BC is a story of perpetual progress and innovation in comprehensions, diagnosis and treatment of this disease (Berg *et al.*, 2012).

BC is a kind of complex disorder with several risk factors associated such as genetic factors. Mutations in several genes have been observed to be associated with an increased risk of BC. BRCA1 and BRCA2 are the important genes in this regard. Mutational changes in this gene can increase a person's risk of developing the BC by up to 80%, as well as it increases the risk of ovarian cancer (Kuchenbaecker *et al.*, 2017). Another kind of gene associated with an increased risk of BC is PALB2. The germline mutation in PALB2 can lead towards predisposition towards BC (Antoniou *et al.*, 2014).

Cytidine Deaminase (CDA) is also an important gene in this regard which encodes an enzyme that is involved in pyrimidine salvaging process. It catalyzes the hydrolytic deamination of cytidine and deoxycytidine to products such as uridine and deoxyuridine (Urbelienė *et al.*, 2023). As a result of this reaction CDA also liberates ammonia (NH_3). It is also observed to be involved in metabolism of several chemotherapeutic drugs that are used to treat cancer, including cytarabine. Single nucleotide polymorphisms (SNPs) of CDA have been observed which change the enzymatic activity and affect the metabolism of cytarabine (Banklau *et al.*, 2010). The catalytic site of CDA and its function is shown below in Figure 1.2.

The zinc element plays a vital role in catalysis. The special feature of the CDA is that the active site of one monomer is built from residues coming from the other 3 subunits through a complex set of inter-subunits

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interactions (Vincenzetti *et al.*, 2008; Micozzi *et al.*, 2010) Its expression is found higher in placenta and liver, whereas, low levels were analyzed in lungs and kidneys. On the other hand, no expression was observed in heart, brain and muscles. Higher CDA activity was reported in liver and spleen, whereas moderate activity was observed in lungs, kidneys and intestinal mucosa. Moreover, no appreciable amount of the enzyme was found in muscles and bones (Miccozi *et al.*, 2014). It has been studied that high CDA activity may degrade the analogue to inactive the metabolites, thus reducing the effectivity of drugs. On the other hand, low activity of CDA may increase the toxicity, sometimes can prove to be fatal. Hence, a strategy based on stratified dosing linked with CDA activity would lead towards enhanced efficacy (Peters *et al.*, 2014). APOBEC is the family of cytidine deaminase which is commonly expressed in mammalian cells. Among this family the subtype APOBEC3B is found to be the major source of mutagenesis in breast cancer (Cortez *et al.*, 2019).

It has been observed in a study conducted in France that CDA increases in the blood of BC patients. Patients with hematological malignancies have reduced levels of CDA, leading towards extreme conditions of toxicity. Similarly, its deficiency has been reported to be involved in genetic instability. The research came out with the conclusion that CDA activity was indicated higher in the serum of BC patients thus highlighting its significance and impact of chemotherapy in BC patients (Buhagiar *et al.*, 2022). In another study, CDA expression was observed to be enhanced in patients suffering from Chronic myeloid leukemia (CML), Overall, the study indicated that gene silencing of *CDA* could inhibit CML that could serve as potential for drug designing (Wei *et al.*, 2018). Moreover, mutagenesis of CDA type APOBEC inhibited the BC growth by stimulating the antitumor immune response mediated by T-cells. Hence, it can be concluded that CDA expression may be dependent on type of cancer patient suffering (DiMarco *et al.*, 2022). In addition, downregulation of *CDA* expression was observed in 60% of cancer cells (Mameri *et al.*, 2017). Furthermore, CDA drives the metabolism of epigenetic nucleosides reveals the therapeutic approach in cancer. The study revealed the extension of recent knowledge regarding nucleotide salvage pathway by showing the metabolism of the oxidized genetic bases, and suggested therapeutic options such as pancreatic cancer which has high expression of *CDA* and are resistant to treatment with other cytidine analogues (Zauri *et al.*, 2015). Keeping in view importance and significance of CDA, this study has been designed to explore the patterns of cytidine deaminase activity in the serum of BC patients on the basis of substrate utility and comparing with control group.

Pancreatic Adenocarcinoma (PDAC) is a kind of disorder with no cure that may soon rank to second cause of death worldwide. Elevated expression level of *CDA* have been studied in PDAC and proves to be a vital factor in tumor progression and metastasis. In PDAC cells CDA prompts DNA synthesis by increasing the stability of replication fork, indicating that CDA is nuclear and located at DNA replication fork. Hence, it depicts that CDA overexpression is involved in regulating stress levels in PDAC cells, a novel approach that can be utilized for therapeutic purpose (Lumeau *et al.*, 2021). Hence a more comprehensive approach is required to find all the possible factors which associate CDA with the deadly progression of breast cancer so that a novel and therapeutic approach can be discovered in future to aid in reducing harmful effects of breast cancer.

MATERIALS AND METHODS

Ethical Statement

After the approval of Ethical Review Committee (ERC-118-2022-; 11-11-2022) and Institutional review board (IRB-415-2023-; 19-01-2023) of Forman Christian College (A Chartered University) this study was conducted.

Study Design

Case-control study.

Study Duration

The study is 9-10 months in duration.

Study setting

School of Life Sciences in Molecular Diagnostic Laboratory, FCCU (A Chartered University).

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Inclusion criteria

- 20 to 70 years old breast cancer patients undergoing chemotherapy, radiotherapy and hormone therapy.

Exclusion criteria

- Untreated patients
- Patients with cysts and abscesses.

Sampling

The clinical sampling involves patient with a certain disease or a condition. The generalizability of clinical sampling includes multiple factors associated with internal and external validity of research methods. The BC patients along with some other cancer patients at Mayo hospital Lahore and Jinnah hospital Lahore, with their consent, were selected and their 5 ml blood was withdrawn by trained medical staff at the facility. The population selected for target sampling consisted majorly of BC patients and some patients suffering from other cancers for comparison purpose. Half the volume of blood collected in EDTA coated Vials and remaining blood was collected in gel vials for serum extraction process (Elfil & Negida, 2017). The samples were transferred to the university laboratory for processing on the same day and were processed according to the procedure shown in Figure 3.1.

RNA extraction

RNA extraction was done according to the protocol described in Biorepository, (n.d.). The TRIzol® method is a general and a recent method for RNA extraction process. TRIzol® actually preserves the RNA molecule by inhibiting the function of RNase enzyme. It consists of monophasic solution of phenol and guanidium isothiocynate that solubalizes biological molecules and denature protein. This method is particularly useful where solution contains endogenous RNA and its seperation is impractical. The procedure is described as follows:

1- 500 µl of blood and 750 µl of TRIzol® were taken into an eppendorf tube. All of these eppendorf tubes were kept on ice. The tubes were vortexed for homogenization and kept at the room temperature for 5 minutes of incubation.

2- 250 µl of chloroform was added to each sample and mixed by hand gently then vortexed. Samples were kept at the room temperature for 5 to 10 minutes of incubation.

3- Samples were centrifuged at 16060 g for 15 minutes at 4°C.

4- Now as centrifugation was done and three layers were produced, the upper one was an aqueous layer that contained our desired RNA, a middle layer containing DNA and denatured protein, and the lower one consisted of organic phase.

5- The samples were kept on ice and then an aqueous layer was isolated and transferred into new Eppendorf.

6- 500 µl of chilled Isopropanol was added to each tube and gently mixed with hands. Incubation was done for 10 minutes at room temperature.

7- Samples were centrifuged at 18626 g for 15 minutes at 4°C.

8- Supernatant was discarded and steps for washing were taken.

9- The pallet was washed by adding 200 µl of 75% ethanol and centrifuged at 6082 g for 2 minutes. Ethanol was discarded. The washing step was repeated at least 2 times. The tubes were left open for air drying for 5 to 10 minutes. Then, 30 µl of nuclease-free water or TE buffer was added to store RNA.

Quantification of RNA

RNA quantification was performed with the aid of Nanodrop machine, which indicates the average RNA concentration in ng/µl. For conducting this procedure, 1µl diluted sample of extracted RNA was utilized in order to obtain the absorbance ratio of 260/280. An optimal absorbance value of RNA is declared as 2.0. Nanodrop values ascertained at a ratio of 260/280 absorbance values for the patients and controls are given below in Table 3.1.

Gel Electrophoresis

The gel electrophoresis was done according to the procedure described by (Lee *et al.*, 2012). It is based on the pore size and concentration of gel, the greater is the concentration of gel, the smaller is the pore size of gel. 1.5 % gel is made with the help of

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1x TAE buffer and then placed on Casting tray. Gel is run for 80 volts for 40 minutes which was then visualized on Gel doc for band analysis. The procedure is described as follows:

- For preparation of 1.2% Agarose Gel, 100ml of 1X TAE Buffer was prepared from the stock of 50X TAE Buffer.
- 1.2g of agarose powder was weighed.
- In 100ml of 1X TAE Buffer in conical flask, 1.2g of agarose was added.
- Mixed it well by stirrer, then heated in microwave oven for 2 minutes.
- It was then allowed to cool down at room temperature just for 1 minute.
- 2 µl Safe-Green™/Ethidium bromide as a loading dye was added in it.
- The solution was poured into gel tank to solidify the gel and fixed the combs into the solution.
- After 25 minutes, PCR products and ladder (1 KB), were loaded into the wells.
- The gel was operated at 80V for 40 minutes.
- Products were visualized under UV light source such as Gel doc.

c DNA synthesis

cDNA synthesis was executed by utilizing Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit #SM0311. It can be used for PCR or real-time PCR as a template. It was performed according to the procedure described by (Li *et al.*, 2012).

Following procedure was used for cDNA synthesis with the help of the mentioned kit. Before starting the procedure, the reagents of kits were thawed and centrifuged properly and placed on ice.

In autoclaved, sterile PCR tubes, following reagents given in Table 3.2. were added while keeping them on ice throughout the procedure.

The prepared mixture was centrifuge for short spin for proper mixing. For oligo dT primer-based cDNA synthesis, PCR tubes containing reaction mixture were subjected to undergo incubation at 42°C for 60 minutes. For random hexamer primer-based cDNA synthesis, the reaction mixture undergoes incubation at 25°C for 5 minutes which was further followed by 60 minutes at temperature of 42°C. The reaction was stopped by heating it at high temperature of 70°C up to 5 minutes. Then the cDNA was stored at -20°C for next experiments or stored at - 80°C for longer period of time.

Polymerase Chain Reaction (PCR)

Gene specific primers of CDA were used for the quantification of data using PCR. The Primers were designed using UCSC genome browser keeping in view melting temperature (Tm) and GC content. The Order of gene specific primers was placed on Eurofins Genomics LLC. They were stored at -20°C. The names of gene specific primers coupled with their sequences are listed below in Table 3.3.

The Polymerase chain reaction was performed in sterile PCR tubes. Premix was prepared sufficient for intended use.

Primer optimization

The PCR has been used in molecular laboratories for the amplification of target DNA sequence. PCR is being used in numerous research purposes such as diagnostic and forensics. A good PCR product depends upon amplification of specific DNA segment in an optimized condition (Ariati *et al.*, 2021).

Primer optimization was done at the annealing temperature of 60°C for both the housekeeping gene *ACTB* and the gene of interest *CDA*. After providing specific conditions of PCR, gel electrophoresis was applied to observe the product sizes as described by (Asif *et al.*, 2021; Mikeska & Dobrovic, 2009).

Normalization process Using ß-Actin Primer

During the whole process, PCR tubes were kept on ice and following reaction mixture was prepared. The reaction was performed with specific quantities of components as described below in Table 3.4.

The Lyophilized state of primers was received. 100 µM master stock of primers was prepared using nuclease free water. For working solution, it was further diluted to 10 µM. It helps in decreasing the chances of contamination of master stock and the optimum PCR conditions of *ACTB* and the gene of interest *CDA* are shown in Table 3.5.

The analysis of PCR products was done through gel electrophoresis. 1.5 % gel is made with the help of

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1x TAE buffer and then placed on Casting tray. Gel is run for 80 volts for 40 minutes which was then visualized on Gel doc for band analysis.

Serum Extraction

The serum contains the remaining liquid part of blood after cells and clotting factors have been removed (Truck et al., 2009). Since it contains our gene of interest in expressed form, it is analyzed and extracted. The total protein content (TPC) will be calculated here after going through the process of Bradford assay. The serum of blood samples was extracted according to the protocol described as follows:

- Take 3ml of blood in a gel vial.
- Centrifuge it at 3421 g for 20 minutes.
- Yellow layer at the top indicates our serum of interest. The serum at the top is isolated and transferred to Eppendorf tubes which is the stored at -80°C for future use.

Bradford assay

Bradford assay is used for evaluation of TPC in serum samples and compared it with the standard curve for determining the unknown concentration. The results for OD and concentrations of known samples is shown in table in results and analysis section. The procedure of the assay is described below (Mehata & Dahari, 2020):

- The BSA 1 mg per ml was prepared to make standard solutions of known concentrations like 10 µg/ml- 100 µg/ml as shown in figure 3.2.
- To make Bradford reagent, Coomassie brilliant blue (G-250) was prepared by adding 10 mg dye in 5 ml of 95% ethanol and then 10 ml of 85% phosphoric acid is added to it.
- The total volume was raised to 100 ml and filtered through what Mann filter paper
- The solution was stored in amber colored reagent bottle under dark conditions at room temperature.

Activity of enzyme:

The enzyme activity was measured through the procedure described by (Buhagiar- Labarchède *et al.*, 2022).

- The Buffer solution was made that consisted of 0.07 M potassium dihydrogen phosphate, and 0.07 M disodium dihydrogen phosphate, adjusted to Ph 7.5 for the enzyme reaction.
- The standard solution was diluted with
- 0.1 ml of sample was taken from each of the prepared standard solutions and dissolved in 5ml reagent and incubated for 5 minutes.
- The OD value of all the concentrations were taken at 595 nm from spectrophotometer and standard curve was formed.
- Keeping in view known concentration, the unknown sample concentration is also calculated through standard curve.

Ammonia testing Principles:

The molecular feature of CDA is its ability to deaminase cytidines and deoxy cytidines into uridines and deoxy uridines, respectively by hydrolyzing amine moiety to ketone and ammonia (Chen et al., 2016). The deamination process which releases uridines and ammonia is very important with aspect of maintaining the balance between ammonium pool and regulating the RNA and DNA synthesis. Since ammonia is released in the process of catalysis, ammonium is tested by adding cytidine as a substrate in our sample of interest and blue color product called indophenol is formed.

Standard solution:

The protocol for standard solution of ammonium chloride was made according to the protocol described by Okamura et al., (1993). The standard solution of ammonium chloride was made by adding 5.135 gram of Ammonium chloride in 25 ml of distilled water and this solution was diluted with potassium dehydrogenase (pH 7.5), to 1:1000 dilution. This is equal to 40 units for 16 hours of enzyme activity. One unit of enzyme activity was considered to produce 10^{-4} µmol of ammonia in one min per ml of serum. A standard blank was also prepared by mixing buffer and distilled water in the same ratio as above.

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the buffer to the units as 20, 10, 5, 2.5 and 1.25 U as shown in Table 3.6.

- The different enzyme units from the stock solution were taken and raised to the final required volume, so that their final concentrations became equal to the respected final unit.
- 50 µl of serum was placed in two tubes, one was used for sample and the other was employed for sample blank.
- Then, 200 µl of water was added in test tubes kept as blank.
- 200 µl of cytidine containing 1.4mmol/ml solution was added in the other tubes. All the tubes were capped and incubated for 16 hours at 37°C in incubator.
- After the required time, the tubes were taken out from incubator. For the precipitation of serum proteins, 100 µl of sodium tungstate (10%), and 100 µl of 1 N H_2S0_4 solution was added to all tubes.
- After the thorough mixing, centrifugation was carried out at 2270 g for 5 minutes and 500 µl of supernatant was added from the tubes to the new tubes.
- The liberated ammonia was detected by enzyme reaction, by addition of 700 µl of phenol solution.
- The phenol solution was made by adding 12 g of phenol, 60 mg of sodium nitroprusside, 900 ml distilled water.
- The hypochlorite solution was made by adding 35.4 gram of hypochlorite, and 1000 ml distilled water.
- After the addition of 1 ml hypochlorite, the mixing was done, which was then incubated for 30 minutes at 37°C.
- The solution was then transferred to the cuvette and its OD value is observed at 630 nm against distilled water.

3.2 Data analysis

The data was analyzed through Excel and SPSS software.

RESULTS

4.1. Demographic data

The data was obtained from the consent of 35 patients and 10 controls. Expression analysis of CDA gene and protein activity of its enzyme were analyzed for all the samples. Based on the ongoing treatment protocol, patients were classified in different groups with 32 patients undergoing chemotherapy, 17 patients undergoing radiotherapy and 21 patients undergoing hormone therapy. A breakdown of demographic data is shown in Table 4.1. Characteristics of different patients are shown according to age, gender, types of treatment and tumor grading. The results of patients obtained were then compared with the results of controls to see how the CDA gene and its enzyme are functioning differently in case of breast cancer patients. The data of patients was divided as below:

Table 4. 1: Characteristics of cancer patients.

Characteristics	**Patients (n)**
Number of patients	35
Median age, years	50
Range	27-75
Sex	
Male	4
Female	31
Disease	
Other cancer patients	9
Breast cancer patients	26
Radiotherapy	
Yes	17
No	18

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Chemotherapy	
Yes	32
No	3

Hormone therapy	
Yes	21
No	14
Tumor grade	
I	8
II	12
III	15

4.2. Extracted RNA

Extracted RNA was analyzed by the procedure of gel electrophoresis. For this purpose, 1.2% gel was prepared and bands were visualized under a UV transilluminator as depicted in Figure 4.1 and Figure 4.2.

4.3. Visual analysis of *β-actin* expression in control group

After RNA extraction from the controls and patient samples, then complementary DNA (cDNA) was synthesized using the ThermoScientific Revert Aid Strand cDNA synthesis kit. For validating the transformation of RNA into cDNA, expression of β-actin was visualized using gel electrophoresis in both control group and patient sample after undergoing PCR. Beta actin is a housekeeping gene which is constantly being expressed in all the cells of the body. Its expression is shown in Figure 4.3. visualize on gel doc. The size of beta actin PCR product came out to be 223 base pairs as analyzed through Insilco PCR on UCSC genome browser as shown below in Figure 4.3. with the help of GeneRuler 1 kb ladder used as molecular marker.

4.4. Visual analysis of *β-actin* expression in patients' samples

Similar procedure was carried out for checking the β-actin expression level in cancer patients by synthesizing cDNA from extracted RNA and then performing PCR for amplification of β-actin products. The products were then visualized on gel doc after undergoing gel electrophoresis as shown in Figures 4.4 and 4.5.

4.5. Visual analysis of *CDA* expression in control group

Keeping in view size of CDA gene according to its sequence obtained from NCBI, the primers were designed whose product was 296 base pairs on UCSC genome browser, PCR amplification of the control group was done with the help of designed primers and gel electrophoresis was performed to observe the results. The Figure 4.6. shows the products of CDA gene in control group which were 296 bp in size analyzed with the help of GeneRuler 1kb ladder.

4.6. Visual analysis of *CDA* expression in patients' samples

CDA expression was also checked in patient samples and the product of 296 bp was observed clearly after performing gel electrophoresis. The results showed that the gene is being expressed in patients as well according to Figures 4.7 and 4.8.

4.7. Expression analysis of *CDA*

The patterns of CDA gene expression were investigated using Real-Time PCR methodology. After that the gene expression data was compiled and organized into graphical formats to ease the data interpretation. To assure the reliability of the primers, a validation process was done through PCR assessment of the control and patient samples followed by gel electrophoresis to ensure the accuracy and precision of the amplified products. The data of patient samples receiving different types of therapies such as chemotherapy, radiotherapy and hormone

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therapy was evaluated and compared simultaneously with the data of controls. The relative *CDA* expression of patients was overall higher as compared to the controls as shown in Figure 4.9.

4.8. Relative expression levels of *CDA* in controls and patients.

The average *CDA* expression was compared among patients and controls. Further the relative expression levels of patients receiving chemotherapy, hormone therapy and radiotherapy were analyzed to see how these therapies are related with the CDA gene expression in patients.

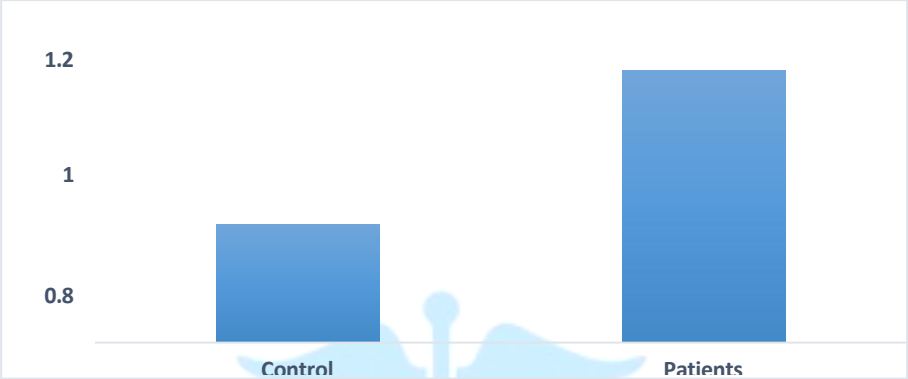

Figure 4.10: Average CDA expression in control group and patients.

The *CDA* gene was showing relatively higher expression levels in patients as compared to control. The results suggest that the elevated levels of *CDA* expression are somehow related with the disease of breast cancer as depicted by above Figure. 4.10.

4.9. Comparison analysis of *CDA* expression among different parameters

Further the data was divided among patients receiving chemotherapy, radiotherapy and hormone therapy to see how these parameters are influencing the average *CDA* expression levels.

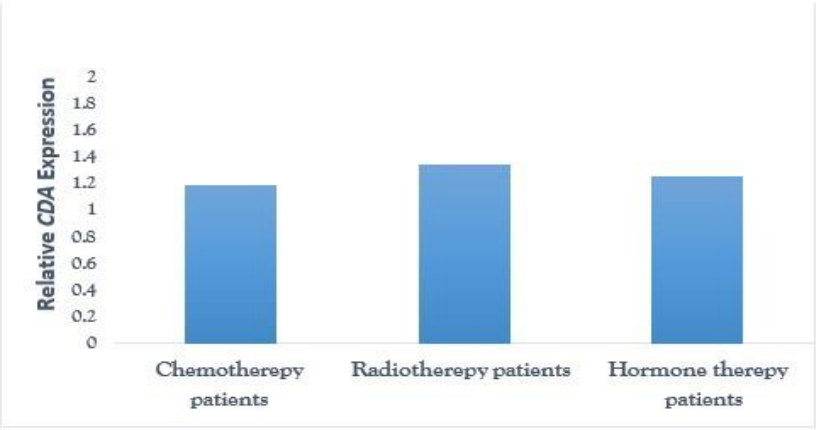

Figure 4.11: Comparison analysis of average *CDA* expression in chemotherapy, radiotherapy and hormone therapy patients

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The patients receiving radiotherapy showed slightly higher expression levels of *CDA* as compared to patients receiving chemotherapy and hormone therapy. These results suggest that the exposure of high energy waves such as gamma rays, x-rays and high beam of electrons and protons may be responsible in stimulating the expression levels of *CDA* as indicated above in Figure 4.11. The data was further divided on the basis of gender to see how the patterns of *CDA* expression differs in case of females as compared to males and to check whether the overexpression of this gene is linked to gender as indicated by Figure 4.12.

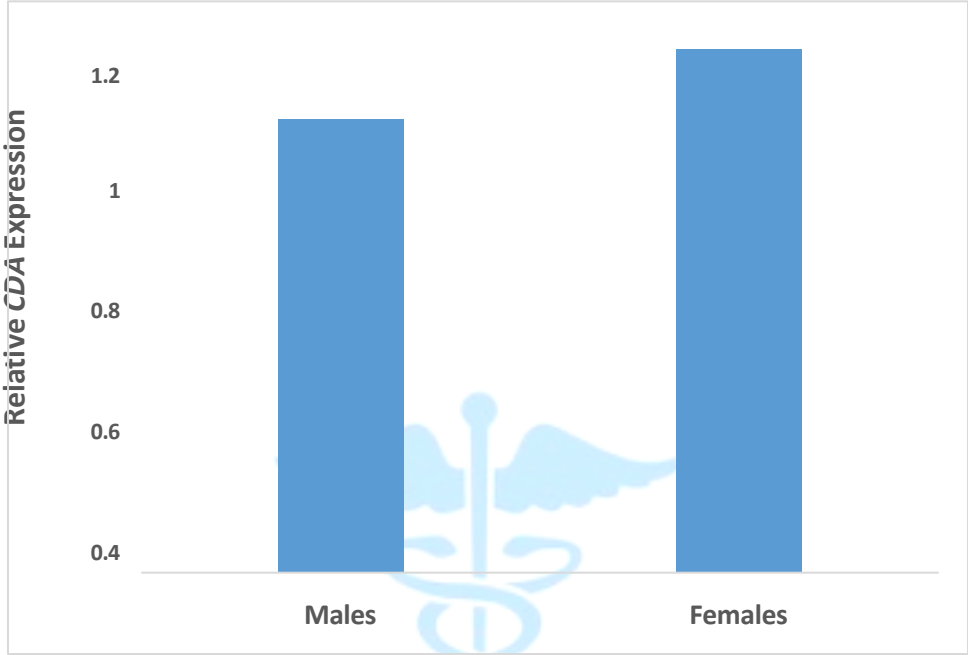

Figure 4.12: Comparison analysis of average *CDA* expression in males and females.

CDA expression levels were found to be elevated in female patient samples as compared to male patient samples which can give us an insight to a new imperative discovery that may be the overexpression of this gene is common in females. To bring more clarity in data, the *CDA* gene expression was analyzed and compared between breast cancer patients and other cancer patients. The results showed that *CDA* expression was relatively higher in breast cancer patients than other cancer patients as given below in Figure 4.13.

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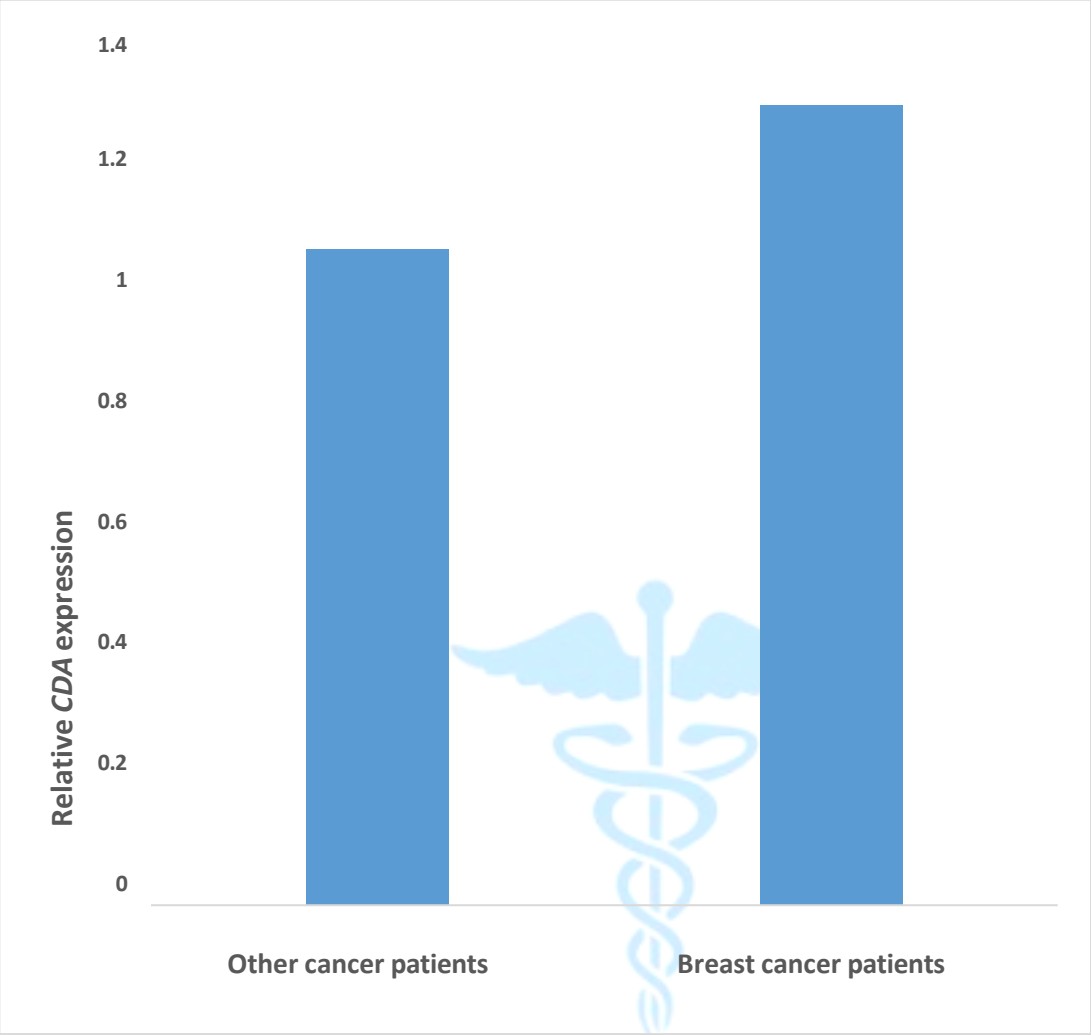
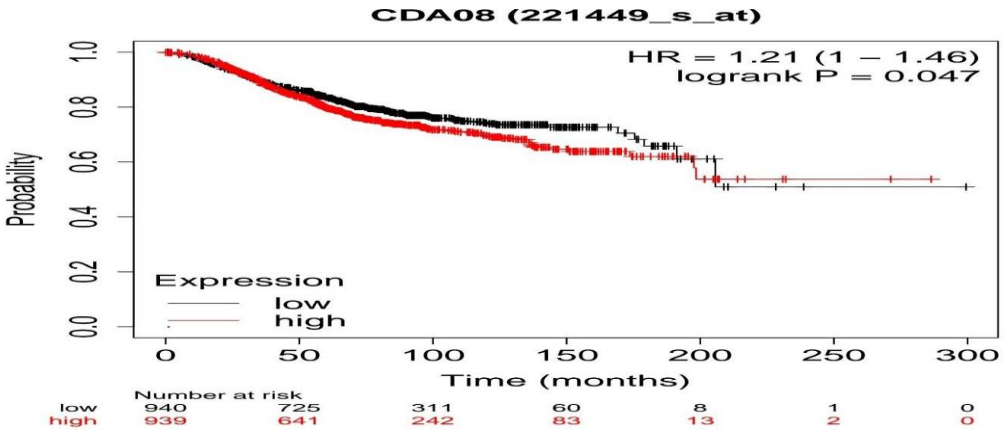


Figure 4.13: Relative CDA expression in



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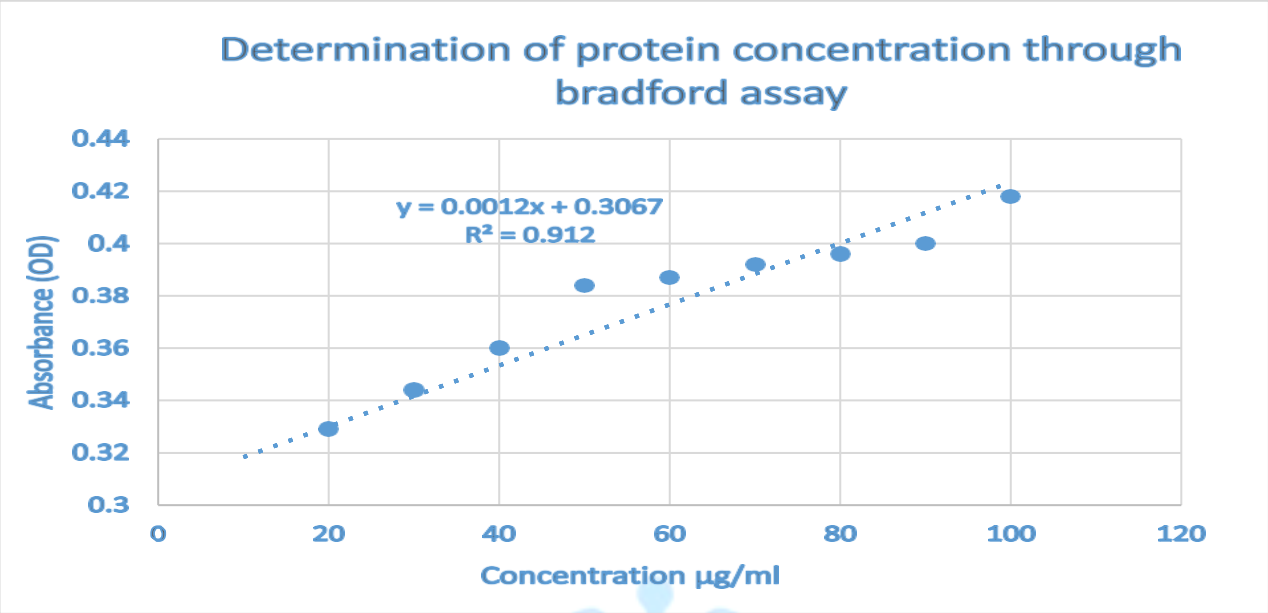

Figure 4.14: CDA Kaplan Meier plot

In above figure, it has been shown how the *CDA* expression is linked with the overall survival probability. This discovery depicts that when *CDA* is expressed at a high rate, the overall survival probability decreases. The significant data related to the *CDA* expression was obtained after excluding the untreated patients. This discovery leads to the idea that patients undergoing some treatment who have high *CDA* expression levels may have a short life span. So, this shows that high expression levels of *CDA* can worsen the effects of breast cancer.

4.10. Bradford test

The Bradford assay is utilized to evaluate the total protein concentration (TPC) in serum samples and compare it with a standard curve to determine the unknown concentration. The Bradford reagent reacts with protein present in samples to form blue color. The greater is the concentration of protein, the greater is the intensity of blue color. The OD values of the known concentrations of the prepared dilutions (µg/ml) were recorded according to the protocol described in methodology section and provided in Table 4.3.

Table 4. 2: OD values of standard solutions

Standard concentration (µg/ml)	OD
10	0.299
20	0.329
30	0.344
40	0.360
50	0.384
60	0.387
70	0.392
80	0.396
90	0.40

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100	0.418

The standard curve of Bradford assay showing concentrations (µg/ml) of the standard dilutions prepared from stock juxtaposed against OD values is plotted as shown below in Figure 4.15. The standard curve derived from the known concentrations of the standard solutions prepared from stock BSA (10-100µg/ml) was then used to calculate the unknown concentration of proteins from the serum samples of controls and patients.

Figure 4.15: Standard curve to evaluate the unknown concentrations from the serum samples

The unknown serum samples were diluted with mili-q water and their OD values were observed in the same method as observed for standards. Concentrations were calculated by the slope of the curve which was y=mx+c. Their TPC was calculated through the formula TPC = Dilution factor (DF) x Concentration (Conc) / sample volume. Triplicate values of absorbance was taken for each sample to obtain maximum accuracy and then average was calculated from the triplicates.

Table 4. 3 Dilution factor, OD values, concentration and Average TPC of 35 patients.

Patients	Dilution (x)	OD	Concentration (µg/ml)	TPC (µg/ml)
1p	100	0.330	19.41	$1.9x10^4$
2p	100	0.378	59.41	$5.9x10^4$
3p	100	0.396	74.41	$7.4x10^4$
4p	100	0.380	61.08	$6.1x10^4$
5p	100	0.387	66.91	$6.6x10^4$
6p	1000	0.329	18.58	$1.8x10^5$
7p	1000	0.326	16.08	$1.6x10^5$
8p	1000	0.387	66.91	$6.6x10^5$
9p	1000	0.381	61.91	$6.1x10^5$
10p	1000	0.399	76.91	$7.6x10^5$
11p	1000	0.334	22.75	$2.2x10^5$
12p	1000	0.337	25.25	$2.5x10^5$
13p	1000	0.339	26.91	$2.6x10^5$
14p	1000	0.325	15.25	$1.5x10^5$
15p	1000	0.401	78.58	$7.8x10^5$
16p	10000	0.345	31.91	$3.1x10^6$
17p	1000	0.386	66.0	$6.6x10^5$
18p	10000	0.315	6.91	$6.9x10^5$
19p	10000	0.399	76.91	$7.6x10^5$
20p	10000	0.380	61.08	$6.1x10^6$
21p	1000	0.393	71.9	$7.1x10^5$
22p	1000	0.381	61.9	$6.1x10^5$
23p	1000	0.401	78.5	$7.8x10^5$
24p	1000	0.410	86.0	$8.6x10^5$
25p	1000	0.409	85.25	$8.5x10^5$
26p	1000	0.322	12.75	$1.2x10^5$
27p	1000	0.346	32.75	$3.2x10^5$
28p	1000	0.323	13.58	$1.3x10^5$

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29p	1000	0.368	51.0	$5.1x10^5$
30p	1000	0.320	11.08	$1.1x10^5$
31p	1000	0.334	22.75	$2.2x10^5$
32p	1000	0.410	86.08	$8.6x10^5$
33p	1000	0.402	79.41	$7.9x10^5$
34p	1000	0.378	59.41	$5.9x10^5$
35p	1000	0.398	76.08	$7.6x10^5$

- The concentration of unknown serum samples was calculated through the excel graph using the equation y= mx + c. Here, y is absorbance, c = 0.3067 (Intercept) and m is slope which is equal to 0.0012.

Those serum samples that were not coming into the range were further diluted with mili-q water to made them fall into the standard range.

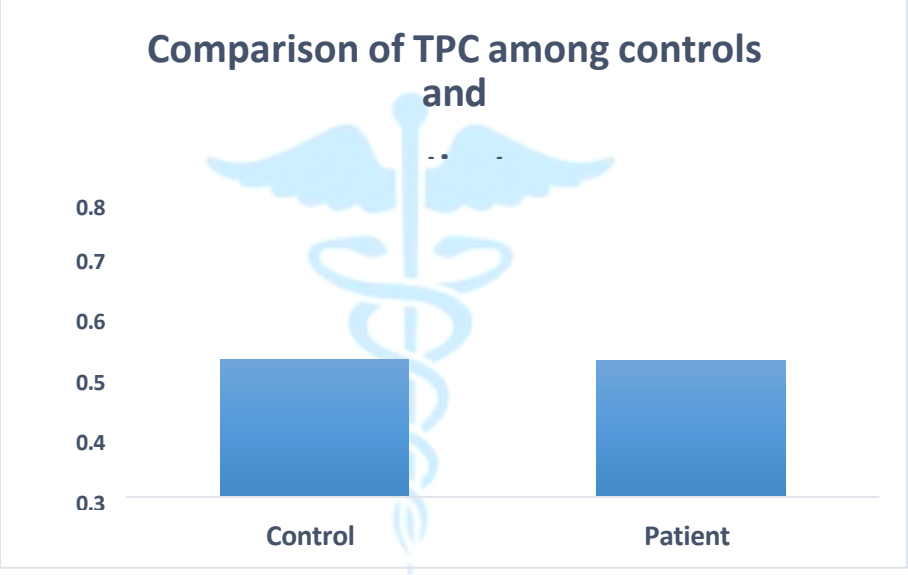

Figure 4.16: Comparison of average TPC between controls and patients.

The average absorbance values of control group came out to be 0.377 which is equal to 5.8 x 10^5 µg/ml (TPC). Compared to average absorbance values of patients that came out to be 0.376 that is equal to 5.7 x 10^5 µg/ml (TPC). There is no such difference between total protein content of control group and patients. Research was conducted to evaluate the TPC in serum of control vs breast cancer patients. It was revealed that TPC in serum of breast cancer and control group came out to be 7.63 ± 0.41g/dL and 6.14 ± 1.84g/dL, respectively (Al-Muhtaseb, S. I., 2014). Thus, there was no significant difference observed between the two groups as shown in our results.

4.11. Standard enzymatic activity units

Starting from the given standard solution of 40 AU prepared from the stock of ammonium chloride solution, further dilutions of 20, 10, 5, 2.5 and 1.25 AU were made. The units for enzymatic activity were given as 1U= 10^{-4}µmol of ammonia produced per min per ml of serum (Buhagiar-Labarchède *et al.*, 2022). OD values were ascertained at 630 nm for the standard dilutions provided in the table below.

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Table 4. 4: OD values of standard ammonium dilutions

Standard enzyme units (AU)	OD values (630 nm)
1.25	0.006
2.5	0.008
5	0.010
10	0.011
20	0.024
40	0.042

Standard curve was then formed according to the absorbance values of standard enzymatic units obtained at 630 nm as depicted below.

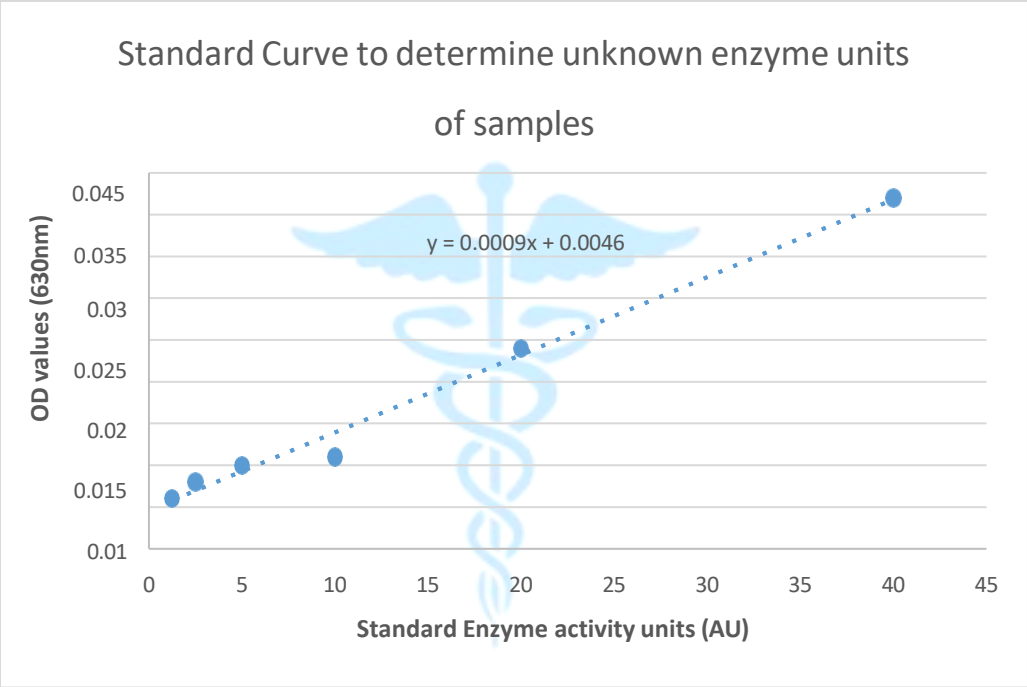

Figure 4.17: Standard curve showing the range of prepared enzymatic units

Units for the enzymatic activity of *CDA* are calculated according to the y-intercept of the standard curve where y-c/m=x. Here y represents the OD values, c=0.0046 (intercept) and m=0.0009 (gradient). Following this method average units were calculated for each sample given in the table below:

Table 4. 5: Average enzymatic units (AU) of control group and patient samples.

Sample ID	Average enzymatic units of *CDA* activity (AU)
C001	33.78
C002	38.73

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C003	26.37
C004	20.4
C005	35.63
C006	21.9
C007	20.8
C008	24.86
C009	24.13
C0010	18.22
S001	23.06
S002	13
S003	12.63
S004	30.76
S005	18.95
S006	14.06
S007	15.6
S008	20.04
S009	31.53
S0010	26.37
S0011	37.47
S0012	35.97
S0013	32.66
S0014	37.48
S0015	27.10
S0016	25.63
S0017	28.93
S0018	31.5
S0019	29.3
S0020	23.06
S0021	36.37
S0022	31.5

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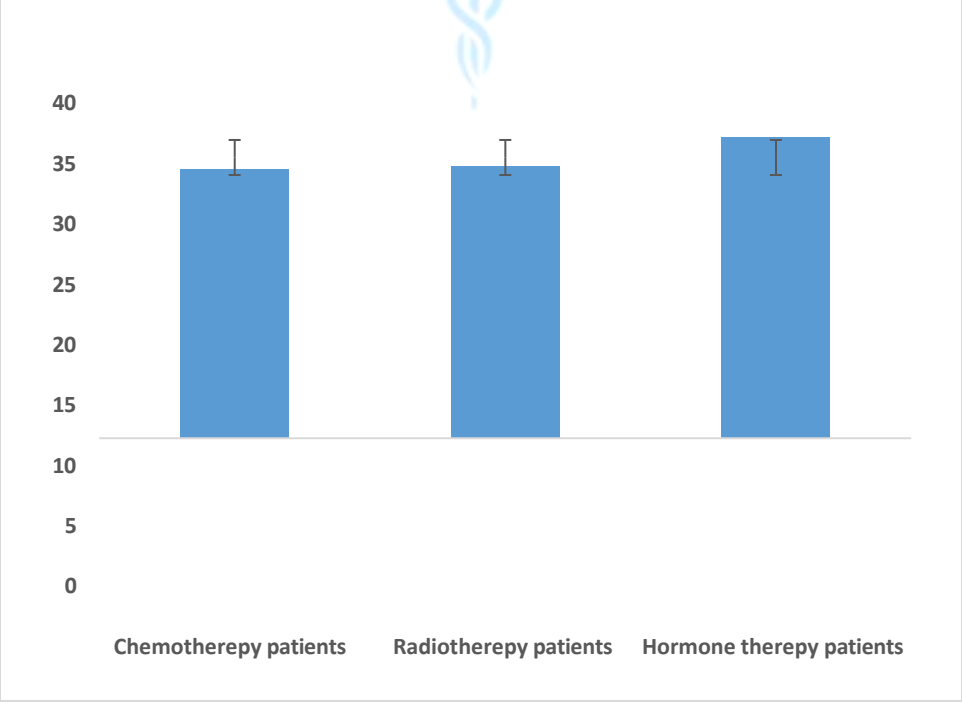
S0023	21.2
S0024	36
S0025	35.63
S0026	38.21
S0027	33
S0028	34.16
S0029	33.76
S0030	36.74
S0031	37.85
S0032	30.76
S0033	34.13
S0034	30.4
S0035	36.74

4.12. Analysis of enzymatic activity among various parameters

Enzymatic units obtained for each control and patient sample were then analyzed. The enzymatic activity of patients undergoing different treatments was compared as shown in Figure 4.17. The enzymatic activity was highest in patients undergoing hormone therapy which suggests that the high activity of CDA is linked with hormonal imbalance inflicted during this treatment.



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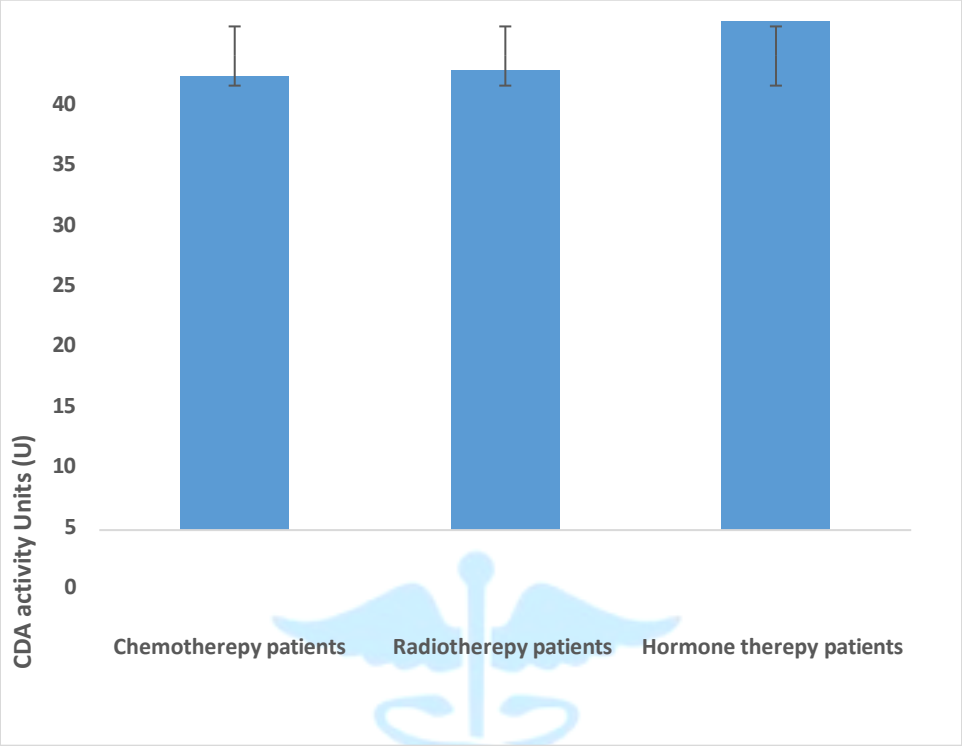

Figure 4.18: Enzymatic activity of CDA among patients receiving different therapies.

Then the enzymatic activity was then compared between control group and patient samples to see how the enzymatic activity is different in both groups as shown below in Figure 4.18. The CDA activity was found out to be high in patients as compared to controls which leads to a new discovery.

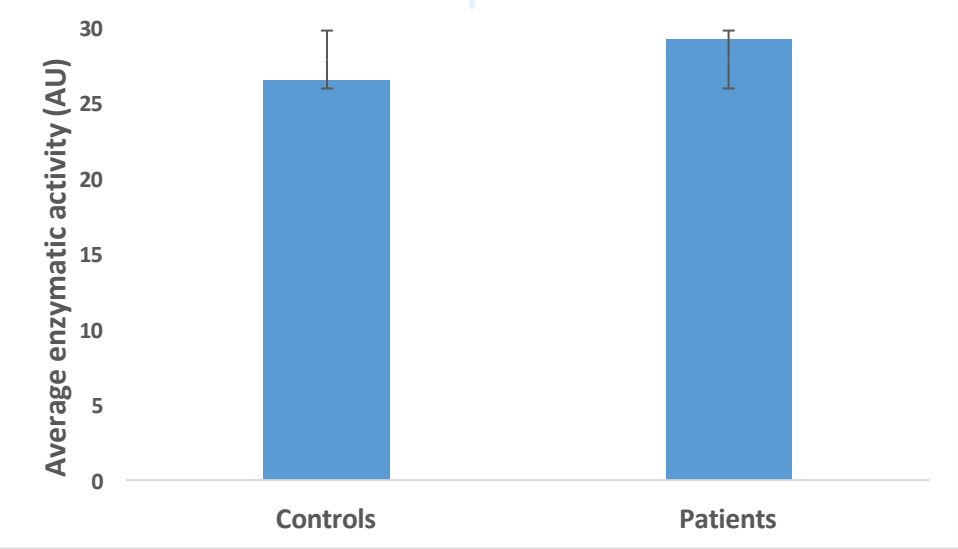

Figure 4.19: Comparison of CDA activity between patients and controls.

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Comparison of average CDA activity among high tumor grade patients and low tumor grade patients was also analyzed as illustrated by Figure 4.19 and the results showed that CDA activity gets elevated in high tumor grade patients which gives an insight to the idea that enzymatic activity of CDA is directly proportional to the tumor grading. The high levels of enzymatic activity may be associated with the high frequencies of treatments being received in high tumor grade patients or it may be associated with the high stress levels in patients as compared to the controls.

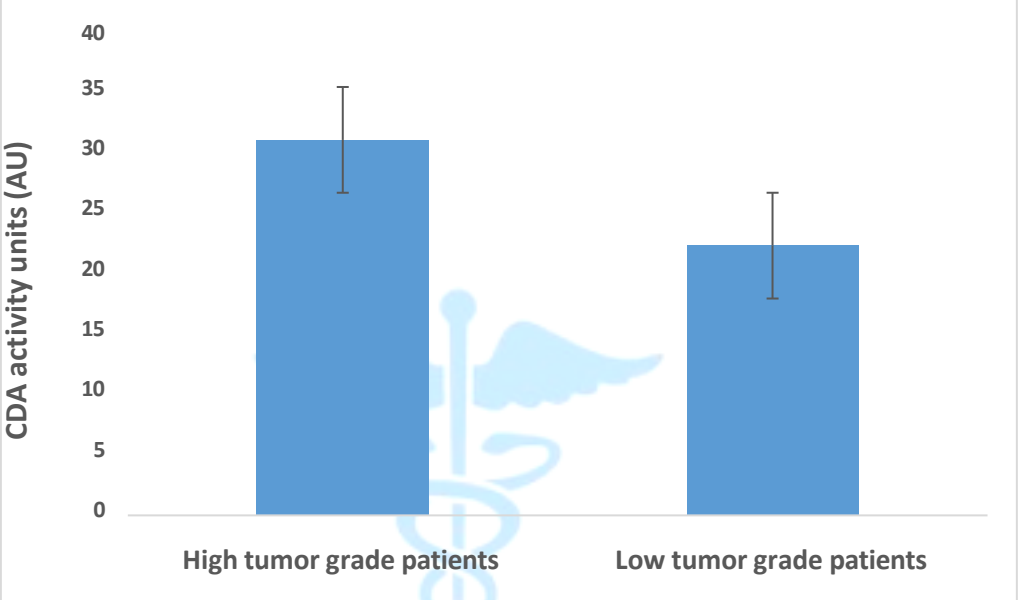

Figure 4.20: Average CDA activity increases in patients with high tumor grades.

CDA activity was also observed among male and female patient samples, and it came out to be high in female patients in comparison of male patients as shown in Figure 4.20. which suggests that may be high expression of CDA is somehow linked with gender.

Figure 4.21: Enzymatic activity of CDA gets elevated in females.

Further to analyze that how the CDA activity is related to the disease of breast cancer, the enzymatic activity units obtained from the samples of BC patients were compared with the samples of other cancer patients having multiple myeloma, papillary carcinoma, rectal adenocarcinoma, Hodgkin lymphoma and ovarian cancer as illustrated by the Figure.4.21. below.

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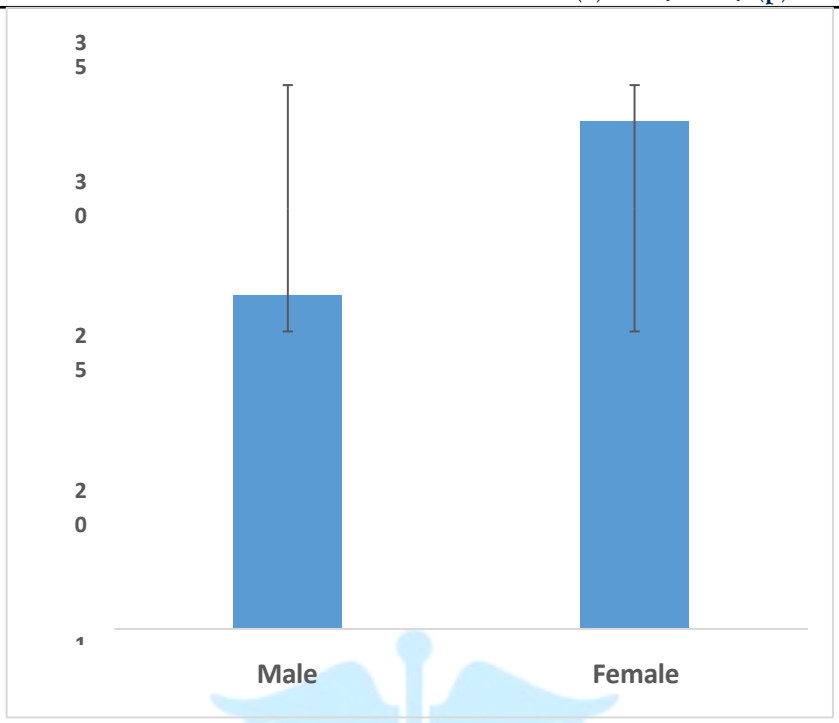

Figure 4.22: CDA activity is high in BC patients.

The results showed that enzymatic activity of CDA is elevated in the BC patients as compared to other cancer patients. This idea leads towards a discovery that the higher activity of the CDA is associated to stress produced in breast cancer patients or maybe it gets elevated due to the therapies being received in breast cancer patients. The results lead us towards therapeutic approach that specific drugs can be produced to suppress the high expression *CDA* gene and to reduce high activity of CDA enzyme to combat the severity of breast cancer.

4.13. Statistical analysis:

Data was analyzed through SPSS software and is explained below:

1. Hypothesis testing is carried out in order to see whether a correlation exists between CDA units (U) and expression levels. Normality between the two variables is tested to find nature of data as depicted in figure 4.22.
   - **Ho:** Data comes from population that follows normal probability.
   - **Ha:** Data comes from population that do not follows normal probability.
2. **Level of significance:** Alpha=0.05
3. **test statistics:** Normality testing analyze> descriptive> explore.

Calculations: Normality testing

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
CDA activity Units (U)	.144	11	.200*	.924	11	.349
CDA units (U)	.227	11	.117	.907	11	.223

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Figure 4.23: Normality tests for CDA activity

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Since, p value is greater than alpha, it is concluded that data comes from the population that follows normal probability distribution. Now, we will go for checking the normality tests for *CDA* expression as well.

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Relative CDA expression Average	.152	10	.200*	.916	10	.328
CDA expression	.157	10	.200*	.929	10	.442

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Figure 4.24: Normality tests for *CDA* expression

Since, p value is greater than alpha, it is concluded that data comes from the population that follows normal probability distribution as depicted from figure 4.23. To find correlation, hypothesis testing is performed.

- Ho: CDA expression and CDA activity are independent
- Ha: CDA expression and CDA activity are not independent

Symmetric Measures

		Value	Asymptotic Standard Error[a]	Approximate T[b]	Approximate Significance
Interval by Interval	Pearson's R	-.035	.113	-.203	.841[c]
Ordinal by Ordinal	Spearman Correlation	-.121	.164	-.701	.488[c]
N of Valid Cases		35			

a. Not assuming the null hypothesis.

b. Using the asymptotic standard error assuming the null hypothesis.

c. Based on normal approximation.

Figure 4.25: Test for finding correlation

Pearson's correlation test is analyzed because data follows normal probability distribution as shown below in Figure 4.24.

Since, p value is greater than alpha, we may not reject Ho, which means no correlation exist between expression level and activity units.

The significance of CDA activity units through SPSS software has been analyzed and hypothesis testing has been applied.

- Ho: average of CDA activity units in male group = Average of CDA activity units in female group

Ha: average of CDA activity units in male group =/= Average of CDA activity units in female group

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Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means							
						Significance		Mean	Std. Error	95% Confidence Interval of the Difference	
		F	Sig.	t	df	One-Sided p	Two-Sided p	Difference	Difference	Lower	Upper
CDA activity Units (U)	Equal variances assumed	.003	.957	-2.615	33	.007	.013	-11.22916667	4.2936750637	-19.96471427	-2.493619067
	Equal variances not assumed			-2.610	2.389	.050	.101	-11.22916667	4.3016054343	-27.12697613	4.6686428001

Figure 4.26: Tests for finding significant difference

As depicted from Figure 4.26. p value is less than alpha, which means Ho is rejected and Ha is accepted. Therefore, average of CDA activity units in male group =/= Average of CDA activity units in female group. Hence, it is concluded that significant difference exists between the CDA activity units of males and females.

DISCUSSION

The main aim of this study was to investigate the potential factors related to the expression of CDA gene and its enzymatic activity which could increase the efficacy and reduce the resistance of treatment modalities in breast cancer patients. To explore the complex dynamics, a comprehensive approach was adopted, involving the extraction of RNA and serum from a well-defined cohort of 35 cancer patients. Among these 9 were the patients having other cancers like multiple myeloma, Hodgkin lymphoma, ovarian cancer, rectal adenocarcinoma, follicular lymphoma, papillary adenocarcinoma and germ cell tumor. Other 26 were breast cancer patients. This patient cohort was carefully juxtaposed against a control group comprising of 10 healthy individuals. Subsequently the extracted RNA was converted into cDNA with the aid of reverse transcriptase kit. Then normalization of the gene expression was accomplished through the incorporation of an important housekeeping gene called beta-actin. Simultaneously, the extracted serum was subjected to Bradford assay to confirm total protein analysis and then was undergone through ammonia testing to ensure the enzymatic activity of CDA with clinical significance.

The gene expression and enzymatic activity data was transformed into informative graphs, visually encapsulating the intricate patterns of gene expression and enzymatic activity. The dataset obtained from PCR was assured by the visualization technique of gel electrophoresis. The dataset resulted was comprehensive and rich with insights into the expression profile of gene of interest, CDA along with its enzymatic activity patterns. The gene was strategically chosen due to its pivotal role in pyrimidine salvage pathway (Lachmann et al., 2013).

The focal point of this investigation was to analyze the potential relation of CDA expression and enzymatic activity with breast cancer. Notably, both the factors were associated with the disease of breast cancer as these were found in elevated levels in patients receiving treatments as compared to the controls. Intriguingly, the results pertaining to CDA expression revealed noteworthy trends. A marginal overexpression of CDA was observed in patients undergoing radiotherapy. A study conducted in Cleveland to evaluate CDA expression in mouse

treated with chemotherapeutic drugs revealed that CDA expression was increased in such mouse. This further supported our study results that displays that expression increases in cancer patients who are treated with different therapies

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as compared to those who have not undergone through it like controls (Mahfouz et al., 2013).

In our investigation, we utilized Kaplan-Meier plot to examine how CDA expression is related with overall survival probability among patients. The emerged results showed that high CDA expression reduces the overall survival probability. The significant results were obtained by excluding the untreated patients and including the chemotherapy patients which gives insights into critical dynamics that CDA expression is triggered by different therapies in patients and can worsen the effects of breast cancer by reducing the life span in patients (Carpi et al., 2013).

Moreover, our data indicates that CDA expression increases in breast cancer patients compared to control group. A finding conducted at the Gansu provincial Hospital in China to analyze the CDA expression level in cancer patients revealed that CDA expression levels in patients' group was enhanced in cancer patients compared to healthy control group (Wei et al., 2018).

The inspection of CDA activity also yielded compelling findings. High levels of enzymatic activity were observed in patients undergoing hormone therapy suggesting a significant link of CDA activity with hormonal disturbance in body. Remarkably, the CDA activity was found higher in BC patients as compared to controls and other cancer patients which further proves a connection between high enzymatic activity of CDA and the administered treatments in breast cancer patients. The prior studies strongly support our findings that CDA activity levels are enhanced in breast cancer patients (Buhagiar-Labarchède et al., 2022). In addition, a study was executed by the scientists at Chemistry department in Iraq to evaluate the CDA activity in BC patients compared with control group. It was expressed that CDA activity was found to be greater in patients affected with BC compared to control group (Altaee et al., 2004). Research executed in Netherlands to study the CDA activity in cancer cells indicated that CDA activity enhanced in cancer affected cells. The enhanced activity was found to be associated with high mRNA levels in cancer affected cells (Bergman et al., 2003).

As far as Total Protein Content is concerned, our study reveals that TPC is nearly same in control group and patient samples. So TPC is not the factor involved in the prognosis of BC. According to the research conducted in Jorden to evaluate the TPC in BC patients compared to healthy control group, it was indicated that total mean of serum protein in breast cancer patients was slightly higher than the healthy control group, but the difference was minor. (Al-Muhtaseb, 2014).

Furthermore, to give our study a more comprehensive approach, normality tests were applied on the dataset of gene expression and enzymatic activity. Both datasets followed the normal probability distribution. For further clarification of results, correlation was found between the two quantitative variables of CDA expression and CDA activity. Our data reveals that no correlation exists between the CDA expression as CDA activity units (U) and both factors are independent of each other. Our study indicates that significant difference exists between CDA activity units of male and female group.

Through this study, the notable parameters of CDA expression and activity were analyzed and their meaningful link with the breast cancer patients was also studied. Higher CDA expression levels in radiotherapy treated patients compared to healthy control group was noted. This discovery can enhance the diagnostic field regarding treatments of breast cancer which do not aggravate the expression and activity levels of cytidine deaminase. The findings of this study illuminate a compelling pattern that both the CDA expression and enzymatic activity exhibited higher levels in case of breast cancer patients. Collectively, the elevated levels of CDA expression and activity contribute towards unfavorable prognosis of breast cancer disease with the worst outcome of reduced life span observed in overall survival probability of patients. Our data reveals that levels of TPC are nearly same in cancer patients and controls. Our results regarding TPC

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could be improved by increasing sample size. Hence, it can be said that CDA gene proves to be potential diagnostic tool in the field of oncology and its activity is also thought to be as a potential biomarker in this field. With the aid of this study, novel drugs can be introduced in future which can specifically target the CDA gene expression for silencing and can lower its enzymatic activity to combat the deadly effects of breast cancer related to this gene.

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