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Correlation of Psychological Stress and Induced Nitric Oxide Synthase (iNOS) Expression in Leukemia

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Abstract: Early life exposure to adverse conditions such as social life issues, economic problems, health issues, death and separation of loved ones produces stress. Stressful life events (SLEs) disturb the healthy quality of life in multiple ways. The biological response to SLE includes the production and activation of stress hormones. It has been reported that adrenaline, noradrenalin, pituitary, cortisol, prolactin, growth and adrenocorticotropic hormones are responsive to SLE. It is observed that under psychological stress, the circulating level of cortisol and norepinephrine (NE) is higher than in normal subjects. Under stress glucocorticoids (GC), neuroendocrine, norepinephrine and catecholamine produce reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS cause oxidative damage and lower antioxidant capacity and protection. Persistent damaged DNA may lead to the initiation of cancer. It is said that the risk factor of immune dysfunction and cancer may be increased under stress conditions by regulation of iNOS. iNOS is most widely studied as it produces large amounts of nitric oxide which affects many vital processes including apoptosis and angiogenesis in leukemia cells. The regulation of the expression of iNOS is important to control the level of reactive oxygen and nitrogen species that can be lethal to the cell and its environment. It is reported that the microenvironment of a cell affects the expression of iNOS. Therefore, it is concluded that different cells breast, colon, esophagus, bladder, lung, oral cavity and prostate might show different expressions of iNOS. Expression of iNOS is higher in tumor cells than in normal controls. Different studies have been conducted to explore the relationship between iNOS and cancer. The aim of this study is to investigate the expression of iNOS in acute lymphocytic leukemia under stress conditions. The study was performed on ALL blood samples under stress and non-stressed conditions. Polymerase chain reaction (PCR) was performed and gene-specific primers were used to see the iNOS expression. The study showed that expression of iNOS is higher in the patient than in control under stress while non-stressed patients showed significant reduction in iNOS expression. Further research is required to validate the importance of psychological factors and iNOS in cancer.

Keywords: Leukemia; Psychological stress; iNOS expression; Cancer

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1. Introduction

Stress is a response to a specific stimulus or stress itself is a stimulus. Early life exposure to adverse conditions such as social life issues, economic problems, health issues, death and separation of loved ones produces stress. Physical stressors such as pathogens and toxins, and psychological stressors such as abuse, trauma, major life events, workplace, family, or neighborhood are observed under SLEs [1]. The response to repeated stress is also determined by the way a person perceives a situation. The biological response to SLE includes the production and activation of stress hormones [2]. It has been reported that adrenaline, noradrenalin, pituitary, cortisol, prolactin, growth and adrenocorticotropic hormones are responsive to SLE. It is observed that under psychological stress the circulating level of cortisol and norepinephrine (NE) is higher than in normal subjects [3]. The nervous system processes the information regulated under stress. Under stress conditions, elevated levels of NE and epinephrine (E) have been reported. There is an association between psychological stress and deregulation or alterations in various neuroendocrine hormones, particularly catecholamines and cortisol [4]. The biological response to stress is thought to be a mediator of the effects of psychosocial factors on cancer progression. The autonomic nervous system and the hypothalamic–pituitary–adrenal (HPA) axis are activated along with several other body systems under stress response [5].

Glucocorticoids (GC) are a class of steroid hormones that are produced by the adrenal cortex. GC binds to its receptor, GC receptor (GCR). Almost every animal cell has GCR. GCs play various roles i.e. they are part of the feedback mechanism as well as interact with many irregular cell responses in cancer. Therefore GCs are used to treat some malignancies [6]. GCs are produced by the adrenal cortex while its receptor is present in the cortisol. GCs are also involved in transrepression and are involved in the upregulation of the expression of anti-inflammatory proteins and the downregulation of proinflammatory proteins [7]. Cortisol is known as a stress hormone. Under stress, it is secreted by the adrenal cortex. Cortisol is one of the important human GCs and is said to be essential for life. Cortisol is known to regulate a variety of important functions such as cardiovascular, metabolic, immunologic and homeostatic roles [8]. High cortisol levels have been reported in high stress while stress reduction is associated with lower cortisol levels. Studies show that neuroendocrine circadian rhythms get disturbed under stress such that it favours tumor growth. GCs act in a bidirectional way i.e. high doses inhibit tumor growth while low doses can stimulate tumor growth [9].

Under stress GC, neuroendocrine, norepinephrine and catecholamine produce reactive oxygen species (ROS) and reactive nitrogen species (RNS). The term reactive oxygen species is used for more reactive oxygen than molecular oxygen (O_2) [10]. The biosynthesis of ROS indicates that it is formed during the metabolism of oxygen by the living system. Superoxide anion ($O^{2\bullet-}$) is formed which proposes the production of ROS. Therefore, increased levels of ROS have been observed in different tumor cells [11]. ROS and RNS cause oxidative damage and lower antioxidant capacity and protection [12]. Persistent damaged DNA may lead to the initiation of cancer. Glucocorticoids bind to the glucocorticoid receptor (GR). It is said that the risk factor of immune dysfunction and cancer may be increased under stress conditions by regulation of iNOS. Nitric oxide synthase is found in three major isoforms i.e. endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) in different tissues [13].

The iNOS regulation is significant in determining its antitumor or proliferative functions. The expression of iNOS is regulated by multiple regulatory mechanisms. Expression of iNOS is higher in tumor cells than in normal controls [14]. It is said that during tumor development, iNOS induces tumorigenic properties in epithelial cells. It has been reported that soon after the onset of stress excitatory amino acids, such as aspartate and

glutamate, are released in stress response and activate NFκB in brain cells. iNOS, NOS-2 and COX-2 genes are responsive to NFκB [15]. Lipopolysaccharides, endotoxin and oxidative stress are involved in iNOS over-expression. Recently, it has been reported that Hsp70 may also be involved in iNOS regulation. Extensive and sustained production of nitric oxide is critical in the regulation of carcinogenesis and progression of cancer cells [16]. iNOS appears to be upregulated in the early stages of cancer development which indicates the importance of iNOS inhibition in chemo-preventive procedures. Several studies have shown the overexpression of iNOS in acute myeloid leukemia, oesophagal cancer and gastrointestinal cancer. iNOS overexpression has been proven to be indirectly involved in the advancement of colon cancer as nitric oxide can enhance the expression of a critical enzyme COX-2 [17].

Nitric oxide produced by iNOS acts both as an anti-tumor and pro-tumor agent. This dual nature is of substantial interest and has been widely debated in cancer biology. According to some *in vitro* and *in vivo* studies, higher expression of iNOS has been reported to possess anti-cancer properties [18]. It was also observed that the dysregulated expression of iNOS depends upon the invasiveness of the tumor. iNOS overexpression or NO overproduction has also been shown to significantly up-regulate several protumor/angiogenic factors and suppress tumor growth and metastases through apoptosis [19]. A study has shown that increased production of nitric oxide facilitates tumor angiogenesis. iNOS induction results in increased vascular permeability and enhanced supply of nutrients to tumor cells thus promoting tumor growth. The clinically characterized as one of the most vascularized cancers, also validated that over-expression of iNOS modulates angiogenesis. The study showed that iNOS inhibition can prove as a good therapeutic strategy for glioma treatment [20].

Leukemia is a malignancy of blood-forming tissues that initiates in the bone marrow and lymphatic system. It is characterized by unlimited growth and rapid differentiation of myeloid or lymphoid progenitor cells. Depending upon lineage and progression rate, it has been categorized into four major classes acute myelocytic leukemia (AML), acute lymphocytic leukemia (ALL), chronic myelocytic leukemia (CML) and chronic lymphocytic leukemia (CLL) [21]. The occurrence rate of leukemia among all cancers is almost 2.7% worldwide. The incidence of leukemia is reported to be 11 per 100,000 persons each year in Pakistan. Acute leukemia is the most common type of malignant tumor found in Pakistani children with a reported rate of 34% [22].

The survival rate for AML is reported to be 50% for patients below the age of 60 years and even lower for adults above that age regardless of many treatments. According to a cohort study conducted in Pakistan, non-specific signs and symptoms are found to be associated with AML. Nitric oxide induces the differentiation of white blood cells in HL-60, a cell line of human myeloid leukemia and affects the expression of many vital genes in these cells. Cytokines and chemokines have been shown to activate iNOS expression, thus causing the production of large amounts of nitric oxide in T-cells as well as in macrophages [23]. Large amounts of nitric oxide induce apoptosis in human leukemia cells and normal bone marrow hematopoietic cells *in vitro*. High levels of exogenous NO mediate cytotoxicity for acute leukemia cells and chronic lymphocytic leukemia (CLL) cells *in vitro*. Many studies on different leukemia cell lines and tumor cells from patients have shown constitutive expression of iNOS and its anti-apoptotic activity [24]. One of the studies confirmed that toll-like receptor 7 (TLR-7) expression in B-CLL is involved in the NF-kB-dependent high transcription activity of iNOS and the increased resistance of tumor cells against apoptosis. iNOS can also lead to the generation of free radical oxygen (O^{2-}) when levels of L-Arginine are low and lead to increased cytotoxicity in macrophages [25]. Long-time production of reactive oxygen species (ROS) under constant oxidative stress causes significant cellular damage and may cause somatic mutations and lead to cancer initiation and progression. According to different studies, overexpression

of NFκB may induce drug resistance and lead to the development of cancer [26]. Therefore, suppression of NFκB limits the proliferation of cancer cells and drug resistance. It is observed that iNOS and certain hormones such as neuroendocrine, glucocorticoids and catecholamines are under the influence of stress. Inhibition of these players can act as a potential therapeutic target in promoting cell survival and treating cancer [27].

Recently inhibition of iNOS in tumor cells has been reported as a healthy treatment for cancer. Stress causes the over-expression of iNOS in tumor cells and metastasizes the tumor cells thus hindering the response to chemotherapy. In this way, stress decreases the resistance to the disease. Therefore, it is estimated that stress treatment can improve the quality of life of cancer patients [28]. The present study will investigate the expression of iNOS in acute lymphocytic leukemia (ALL). This study will determine if iNOS expression is high in ALL patients due to psychological stress and if iNOS inhibition helps in treating ALL. The study will provide insight into understanding how psychological stress is related to leukemia and it will elaborate on how iNOS is linked to stress.

2. Methodology

Diagnosed ALL patients with stress from PIMS hospital, Islamabad were obtained in this study cohort. Fresh blood samples of patients had been collected and stored in EDTA tubes. Blood samples were stored immediately and brought to the laboratory for further processing. ALL patients under stress were included. The chemicals and reagents used for RNA extraction were 70% ethanol, chloroform, isopropanol, RBCs lysis buffer, autoclaved distilled water, TE buffer, and TRIzol®. The PCR conditions were optimized. RNA was extracted using the TRIzol® reagent method. Labeled Eppendorf tubes were centrifuged. The pellet was collected and the supernatant was discarded. 1 mL TRIzol® was added. The cell lysate was transferred to a 1.5 mL Eppendorf tube and 0.3 mL chloroform was added. After 2–3 minutes of incubation, the tube was centrifuged at 12,000 rpm for 15 minutes at 4℃. Two layers appeared in the tube. The upper aqueous layer was separated into another Eppendorf and 0.4 mL isopropanol (100%) was added to the aqueous phase. The Eppendorf was incubated for 10 minutes. After repeating the centrifugation step, the supernatant was removed, and an RNA pellet was observed. 1 mL of 70% ethanol was added. After incubation of 10 minutes, the Eppendorf tube was set for centrifugation at 7,000 rpm for 5 minutes. Ethanol ethanol-containing supernatant was removed and the pellet was air-dried. The pellet was dissolved in RNase-free water and stored at -20℃.

RNA quantification was done using a Nanodrop Spectrophotometer. RNase-free water (1 µL) was used as a blank for the quantification procedure. After optimization with blank, 1 µL of sample was loaded on the sensor of quartz cuvette which was covered with a lid of lid factor 50. The absorbance of RNA samples was measured at a 260/280 nm ratio. Qualitative analysis was followed by gel electrophoresis of isolated RNA samples. 1 g agarose (weighed using a weighing balance) was dissolved in 100 mL of 1X TBE buffer by heating in the oven. After agarose was completely dissolved in the buffer and the solution was clear, 8 µL of ethidium bromide was added. The liquid gel mixture was poured into a gel caster fixed with combs and left at room temperature for solidification. After solidification, the combs were removed carefully to get the wells. Gel was then placed in a gel tank filled with 1X TBE running buffer for loading and running samples.

cDNA was synthesized from extracted RNA extracted with a concentration of 500 ng/µL using a PCR machine. RNA sample to be added in a reaction mixture of 20 µL was calculated by the yield. Add the reagents mentioned in **Table 1** in PCR tubes so that the total volume is raised to 13 µL. Centrifuge the tubes for a short

rotation of 3–4 seconds to mix all the components well. Place the tubes in the PCR machine for 5 minutes at 65°C. Reagents for the annealing step of cDNA synthesis RNA, oligo dT, and autoclaved distilled water with a total volume of 13 µL. Remove the tubes from the machine and chill on ice. After cooling the tubes, add the reagents mentioned in specific amounts to make the reaction volume up to 20 µL in each tube. Reagents for the extension step of cDNA synthesis were 10X RT buffer (4 µL), RT enzyme (1 µL), and 10 mM dNTP mix (2 µL) with a total volume of mixture was 7µL. Place the tubes back in the PCR machine and resume the reaction cycle from step 2. Reaction continues at 42°C for 2 hour and then at 70°C for about 10 minutes for enzyme inactivation. cDNA obtained was stored at -20°C and used further for primer optimization and real-time PCR.

Gene-specific primers for iNOS were designed by an online primer designing tool Primer3Plus. The gene sequence of iNOS was retrieved from the Ensemble Genome Browser. After designing the primers, their binding specificity was checked through the NCBI BLAST tool. Primers were designed by Macrogen (Korea). The primer sequence and product size are given in **Table 1** below.

Table 1. List of forward and reverse primers of iNOS designed using Primer3Plus

Primer	Primer sequence (5'–3')	Melting temperature	GC content	Annealing temperature	Product size
Forward	TGCAGACACGTGCGTTACTCC	61.8°C	57.14%	58.0°C	129 bp
Reverse	GGTAGCCAGCATAGCGGAT	60.2°C	60.00%	58.0°C	129 bp

Primer dilutions up to 10 mM concentration were prepared by dissolving the primer from 100 mM stock solution in autoclaved distilled water in a 1 to 10 ratio respectively. Dilutions were stored at -20°C for use in reaction later. The melting temperature of the primer was calculated by the NEB online Tm calculator and it was 60°C. The annealing temperature was calculated to be 58.0°C. Seven temperatures selected for gradient PCR were 57°C, 58°C, 59°C, 60°C, 61°C and 62°C. PCR (Polymerase Chain Reaction) was performed for optimization of primers. cDNA along with forward and reverse primers of iNOS and other chemical reagents in given quantities were added to 6 PCR tubes. The tubes were given a short span of 5 seconds in a centrifuge machine for thorough mixing of all the components. The final volume of the reaction mixture was 20 µL in each tube.

On 2% agarose gel the amplified PCR products were analyzed. 2 g agarose was added in 100 mL buffer (1X TBE) and boiled in a microwave. Ethidium bromide solution (5.5 µL) was added. 10 µL PCR product and 2.5 µL loading dye were mixed and loaded into the gel well. A 2.5 µL DNA ladder (100 bp) was loaded to analyze the PCR result. Electrophoresis was performed at 70 volts for 1.5 hours and 300 mA in 1X TBE buffer. For visualization of amplified gel products gel documentation system (Biometra) was used.

The quantitative polymerase chain reaction was performed with forward and reverse primers to check the relative iNOS mRNA expression. Reagents mentioned in **Table 2** were used in this reaction. The PCR tube containing a reaction mixture of 10 µL volume was placed in a real-time PCR machine and conditions were set with an annealing temperature of 58°C.

Table 2. Composition of the reaction mixture used for qPCR

No.	Reagents	Volume used/samples
1	cDNA (prepared from 200 ng RNA)	1 µL
2	2X SYBR green	2 µL
3	10 mM Forward primer	0.25 µL
4	10 mM Reverse primer	0.25 µL
5	Autoclaved distilled water	6.50 µL
6	Total volume of the reaction	10 µL

For control, single copy gene (SCG) β-globin was used as an internal control to normalize the expression of iNOS. Data was used to analyze the expression of iNOS. mRNA expression of iNOS was represented in the form of average ΔCT values.

The data was analyzed using a student *t*-test to check the correlation of control and patient samples for expression of iNOS. Results with $P < 0.05$ were considered statistically significant.

3. Results

RNA was extracted from patients obtained from hospitals and control samples using the TRIzol method. Extracted RNA was diluted using RNase-free water. From all the extracted samples, some random samples were selected and evaluated on 1% agarose gel through electrophoresis. The product was visualized in a UV transilluminator as shown in **Figure 1**.

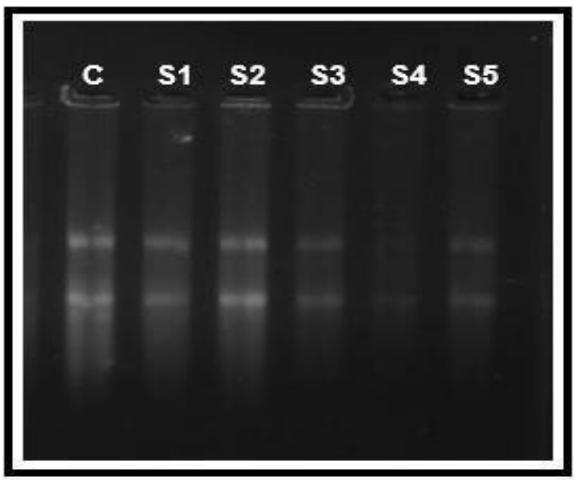

Figure 1. Gel electropherogram of 1% agarose gel showing RNA extracted from patient and control samples of leukemia. Labels from S1 to S5 show treated samples while C is an untreated/control sample.

Polymerase chain reaction was performed for iNOS primer optimization. The calculated annealing temperature was to be around 58°C. Accordingly, temperatures selected for the optimization ranged from 57°C to 62°C. These temperatures were 57°C, 58°C, 59°C, 59°C, 60°C, 61°C and 62°C. cDNA from the normal person was used for primer optimization, and a 1 kb DNA ladder was used to compare the size of the product

obtained. Results for optimization are shown in **Figure 2**.

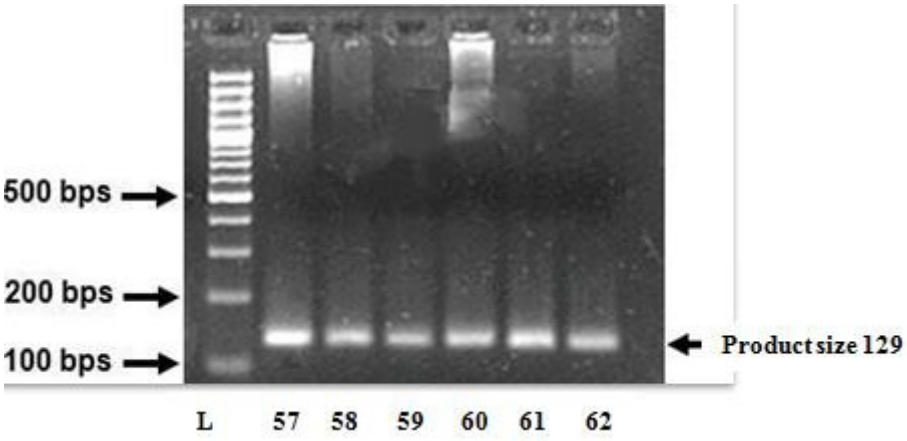

Figure 2. Gel electropherogram of 2% agarose gel for optimization of iNOS primers at different temperatures showing a product size of 129 bp.

After gradient PCR, the annealing temperature of iNOS primer was confirmed by performing PCR at a specific temp of 58°C. Reaction conditions were the same. From confirmation, PCR results it was concluded that 48°C is the annealing temperature for iNOS primers. The result of the confirmation reaction is shown in **Figure 3**.

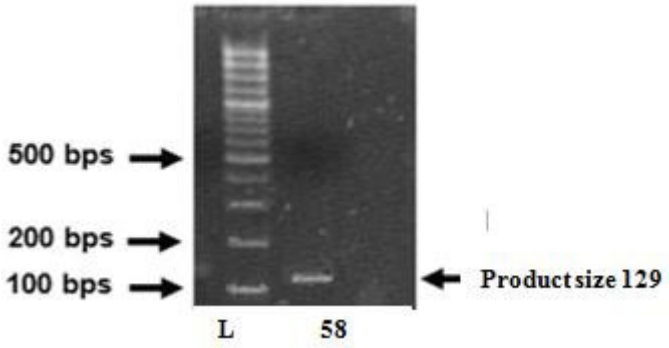

Figure 3. Gel electropherogram of 2% agarose gel for confirmation of iNOS primer optimization showing 129 bp products at 58°C.

Real-time polymerase chain reaction was done to quantify iNOS expression in cancer cells by using gene-specific primers. In cancer cells expression of iNOS is usually high under stressed conditions. Expression of the cancer and control samples was normalized by housekeeping gene β-globin and represented as average ΔCT. The ΔCt values obtained from the real-time PCR were normalized by the ΔCt values of the housekeeping gene beta globin. Expression of iNOS in cancer cells was represented by average ΔCt. Expression analysis in samples shows that iNOS has a significant effect on leukemia.

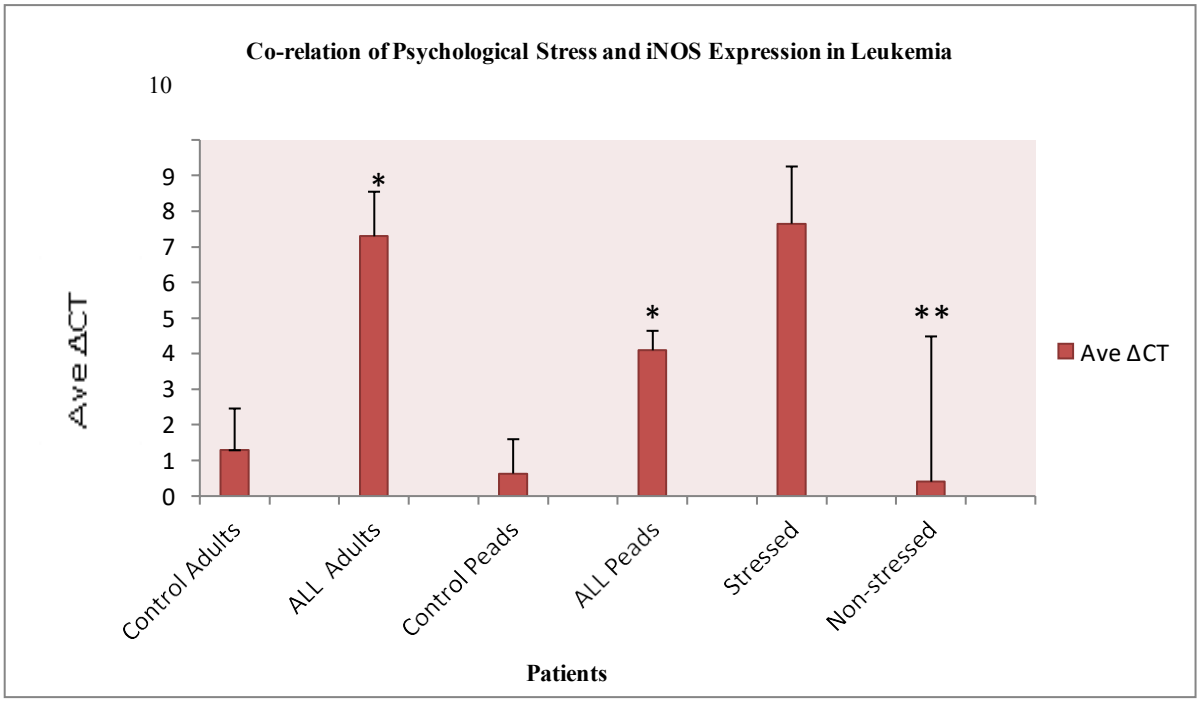

Figure 4. Expression of iNOS in ALL under psychological stress.**P*-value < 0.05 (Significant) ***P*-value less significant

As shown in **Figure 4**, expression is higher in ALL adults as compared to the control. Likewise, a significant increase in iNOS expression was observed in ALL peads relative to the control samples. This analysis indicates that expression of iNOS is increased with an increase in psychological stress, whereas a little non-significant reduction of expression is seen in non-stressed samples.

4. Discussion

Cancer is the leading cause of death all over the world. It is a hidden player in the progression of tumor cells along with many other physiological disorders. Psychological stress induces resistance in tumor cells by over-expression of iNOS in cancer patients. Stressful life events (SLEs) such as social pressure, unemployment, loneliness, fear, anger, death, illness and relative issues disturb the healthy quality of life in multiple ways [29]. The biological response to SLE includes the production and activation of stress hormones. Under stress, ROS and RNS are produced by glucocorticoids (GC), neuroendocrine, norepinephrine and catecholamines. ROS and RNS cause oxidative damage and lower antioxidant capacity and protection. Persistent damaged DNA may lead to the initiation of cancer [30].

The risk factor of immune dysfunction and cancer may increase under stress conditions by regulation of iNOS. Therefore, certain cancers such as breast, colon, esophagus, bladder, lung, and oral cavity show upregulation of nitric oxide (NO). Data indicates that in different cancers such as breast, colon, lung etc. reactive oxygen and nitrogen species can sensitize cancer cells to chemotherapy [31]. Expression of iNOS is higher in tumor cells than in normal controls. It is said that during tumor development, iNOS induces tumorigenic properties in epithelial cells [32].

In the present study viewing the regulatory interactions of psychological stress with ROS/RNS and iNOS,

a pathway was established involving these three factors. Here, ROS/RNS and stress both positively regulate the expression of iNOS in tumor patients. Reducing the level of ROS/RNS and stress can lead towards the inhibition of this regulatory pathway. Data showed that lipopolysaccharide, endotoxin and oxidative stress are involved in iNOS over-expression. According to some studies, IL6 may be regulated by the NF-κB. The inhibition of transcription factors such as IL6 and NFκB can reduce the response of iNOS in lymphocytic leukemia patients [33].

5. Conclusion

It is observed that iNOS and certain hormones such as neuroendocrine, glucocorticoids and catecholamines are under the influence of stress. Inhibition of these players can act as a potential therapeutic target in promoting cell survival and treating cancer. Recently, inhibition of iNOS in tumor cells has been reported as a healthy treatment for cancer. Stress causes the over-expression of iNOS in tumor cells and metastasizes the tumor cells thus hindering the response to chemotherapy. In this way, stress decreases the resistance to the disease. Therefore, it is estimated that stress treatment can boost the standard of life of cancer victims. The present study concluded that therapies need to be found to improve the stress state of cancer patients as patients are showing reduced responses to chemotherapies. It would be an alarming situation when the patient acquires complete resistance toward possible treatments. Therefore, when doctors are trying to diagnose cancer, they should also need to consider the stressful life events of cancer patients. Stress should be considered a serious state, or leading state of illness in cancer patients.

Author contribution

Conceptualization: Assad Ullah
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Writing – original draft: Komal Ghafoor
Writing – review & editing: all authors

Disclosure statement

The authors declare no conflict of interest.

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