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REVIEW ARTICLE

Cancer stem cells: Review of current state and future directions

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Abstract
Cancer is a group of disorders characterized by the abnormal proliferation of cells. Cancer can infiltrate or spread to different areas of the body. Cancer stem cells (CSCs) are small groups of cancer cells that can self-renew and develop into tumors by different signaling pathways. CSCs play a significant role in the development of many types of cancer and are a significant factor in tumor metastasis and relapses. They are usually resistant to the existing treatments primarily focusing on most tumor cells. Recent technological advances have made it easier to study the basic role of CSCs in cancer biology and target them for potential therapeutic strategies. In this minireview, we give a brief overview of recent insights into the characteristics, mechanisms, current treatment opportunities, challenges, and future directions of cancer stem cells and their potential to revolutionize cancer treatment.
Keywords: Cancer stem cells, Cancer biology, Therapeutic potential.

Abbreviations
AML: acute myeloid leukemia; ATP: adenosine triphosphate; CD: cluster of differentiation; CSCs: cancer stem cells; CAFs: cancer-associated fibroblasts; CXCR: CX chemokine receptor type 4; DNMT1: DNA methyltransferase 1; ECM: extracellular matrix; EMT: epithelial-mesenchymal transition; FGF: fibroblast growth factor; HCC: hepatocellular carcinoma; MSCs: mesenchymal stem cells; MDSC: myeloid-derived suppressor cells; PDGF: platelet-derived growth factor; TAN: tumor-associated neutrophils; TAM: tumor-associated macrophages; VEGF: vascular endothelial growth factor.

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INTRODUCTION

Cancer is a group of disorders characterized by the abnormal proliferation of cells that can infiltrate or spread to different areas of the body. Although there have been advancements in cancer therapy, it continues to be a prevalent cause of mortality worldwide. The probability of treating cancers is most significant when they are detected in the early stages and treated with standard methods like chemotherapy, surgery or radiotherapy [1, 2]. However, a significant number of cancers are detected at an advanced stage, by which time they progressed and metastasized to other organs. Despite early diagnosis and treatment of cancers, residual cells persist and can eventually lead to metastasis and tumor recurrence and resulting in a more aggressive form of the disease. There is increasing evidence suggesting that residual cells identified at any stage of cancer development, known as cancer stem cells (CSCs), are responsible for therapy resistance due to their stem cell-like properties [3]. CSCs initially discovered in 1990, are a limited group of cancer cells that possess the ability to self-renew and sustain their stem stem-like properties, as well as exhibit resistance to drugs. The ability to self-renew and specialize into numerous cell types present in the tumor distinguishes them from the majority of other cancer cells [4]. The CSC model provides a conceptual framework for understanding the presence of diverse cell types within a tumor, as it enables the identification of different phenotypes and the maintenance of their population. Tumors lacking stem cells exhibit varying degrees of cellular differentiation, however; they exhibit a high rate of cell proliferation. Extensive research has been conducted on their potential to evade current cancer treatments, since concerns have arisen about their potential to develop into cancer and their suitability for use in therapy. They possess the ability to multiply and become more lethal as a result of their adaptation driven by natural selection to endure in harsh environments [5]. Numerous surface markers, such as CD44 and CD133, and signaling pathways, such as Wnt and notch pathways have significant roles in the regulation of cancer stem cells. Therapeutic treatments, including tyrosine kinase inhibitors, monoclonal antibodies, chimeric antigen receptors (CAR) T cells, and tumor vaccines, have been developed to specifically target these surface markers and pathways of CSCs [2,4].

The intricate molecular components and pathways comprise a complex and dynamic regulatory network that regulates the diverse development and potential of CSCs in the tumor environment. The balance between the mechanisms of self-renewal and differentiation plays a crucial role in the proliferation and progression of tumors. A detailed understanding of this intricate

interplay of molecules is necessary for the progress of targeted therapy, thereby reducing the probability of cancer reoccurrence. Current treatments are costly and mostly ineffective, therefore novel treatments to target CSCs are required. The expanding understanding of fundamental biology has the power to improve existing therapeutic methods in the near future [5]. We summarize the overview of characteristics, mechanisms, and current treatment opportunities of cancer stem cells. We also highlight the challenges and future directions of CSCs implications for revolutionizing cancer treatment.

Discovery of Cancer Stem Cells

The theory about the existence of cancer stem cells (CSC) fundamentally reshapes understanding of tumor biology. It has been proposed that tumors are organized in a hierarchical manner like normal tissues. In the tumor hierarchy, CSCs exit at the top followed by progenitor cells and differentiated cancer cells. These cells are supported by the surrounding tumor microenvironment that drives tumor growth, progression and resistance to therapy. CSCs perform dual functions i.e. proliferation and differentiation. The cancer stem cell theory gained strong evidence from the identification of CSCs in several types of cancer, including leukemia, breast cancer, and brain tumors. Notably, only a small fraction of cells within these tumors had the ability to multiply and become cancerous when transplanted into other organisms. Ernest McCulloch and James Till, two Canadian scientists from the University of Toronto, made groundbreaking discoveries in the 1960s that laid the foundation for the concept of cancer stem cells [6, 7]. They demonstrated the existence of stem cells in mouse bone marrow and proposed the idea that cancer might arise from these stem cells. Their work revolutionized the field of hematology and oncology and paved the way for later research on CSCs. In 1997, John and Bonnet made an important discovery in stem cell research. They identified cells with a high potential to multiply in acute myeloid leukemia (AML) and isolated a specific type of stem cell known as CSCs, which are characterized by the CD34+CD38− phenotype [4]. In 2001, this significant discovery recognized the presence of leukemia stem cells and laid the foundation for the concept of cancer stem cells. CSCs have the ability to self-renew and are responsible for driving the formation and growth of tumors, similar to normal stem cell function [8] but with a specific role in the advancement of cancer. The notion of CSCs has broadened to cover diverse solid tumors [9]. In 2003, Al-Hajj initially isolated CD24−CD44+/low CSCs from breast tumors, which demonstrated notable tumorigenicity in mice. Transplanting a small number of CSCs in breast cancer can result in the formation of a tumor, while transplanting a significantly larger number of normal cancer cells does not have the same effect. Similar findings were obtained from biopsies of human carcinomas from colon carcinomas, brain tumors, and head and neck malignancy. Researchers transplanted CSCs into mice with weak immune systems, and these cells grew into new tumors that were identical to the original tumors. CSCs have also been identified in pancreatic carcinoma, lung cancer, and malignant melanoma. Because of their ability to resist apoptosis, both chemotherapy and radiation are ineffective in destroying majority of the CSCs (Figure 1). Chemotherapy and radiation can decrease the growth of a tumor, but only the resilient cells can survive. This is why remission is frequently followed by a particularly aggressive recurrence. CSCs possess three key survival strategies that enable them to resist cancer treatments including they can enter a dormant state, employ detoxification mechanisms to remove harmful substances, and activate protective pathways that prevent cell death. The prospective cancer treatments must specifically focus on tumor stem cells to successfully combat cancer [5].

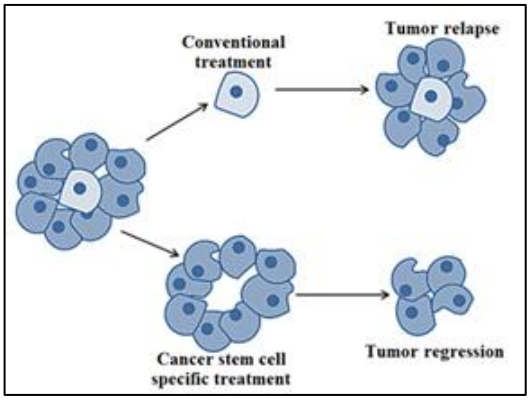

Figure 1: Cancer Therapies. Tumor-relapse after conventional cancer treatment (chemotherapy and radiation therapy) due to the presence of cancer stem cells. Treatments that target cancer-specific stem cells produce tumour regression.

Characteristics of Cancer Stem Cells

Cancer stem cells possess some unique features including self-renewal, differentiation, proliferation, metastasis, angiogenesis, and tumorigenesis. One of their characteristics is the ability to divide and renew them, making them capable of growing indefinitely. This property is often assessed using *in vitro* assays, such as limiting dilution assays or sphere formation assays, where CSCs can grow as non-adherent spheroids in suspension culture, indicating their stem cell-like properties. CSCs have been reported to have increased resistance to conventional treatments such as cytotoxic drugs and irradiation that are known to be effective in proliferating cells. This resistance occurs due to multiple factors that include the upregulation of such proteins as drug efflux pumps, which include the ATP binding cassette transporters, enhanced DNA

repair capacity, and the ability to exit the cell cycle and become dormant in response to administered treatments. Additionally, CSCs are multipotent as they can differentiate into various specialized cell types that constitute the tumor, thus contributing to its heterogeneity. This differentiation potential is crucial for tumor adaptation and survival in response to environmental stress. Cell surface proteins including CD44 and CD133 have been used for the selective isolation of CSCs. However, differences in marker expression based on tissue type and tumor stage limit the general applicability of targeting cancer stem cells, making it essential to develop more specific and novel approaches for effective treatment [10].

Molecular Mechanisms of Cancer Stem Cells to Develop into Cancers

The CSC niche consists of several components that keep them in an inactive state. Niche also controls the adaptability and inactivity of the cells by activating multiple essential molecules, processes, and signaling pathways. The mechanisms and pathways in the CSC niche represent the comprehensive network of all component and cells interactions inside the niche.

Signaling Pathways Regulating the CSC Niche

The regulation of cancer stem cells may be complex, which encompasses multiple signaling processes and external microenvironments [11]. Some of the important signaling pathways that have been implicated in determination of CSC properties include Wnt/ β-catenin/TCF, Notch, and Hedgehog signaling pathways. These pathways are involved in self-renewal catenin to move into the nucleus, where it triggers the expression of genes involved in cell growth, self-renewal, and proliferation. Another key pathway implicated in regulation of CSCs and their properties is the Notch signaling pathway, which is essential for the balance between self-renewal and the process of differentiation in CSCs. Overall, this pathway promotes the stemness of these cells. The Hedgehog pathway has been reported to play a role in maintaining self-renewal of CSCs in various cancers. It promotes tumorigenesis and metastasis of these cells. CSC function and activity is influenced by its surrounding stroma. The stroma includes stromal cells, immune cells and the extracellular matrix. CSCs are important in that they can also directly interact with neighboring cells, and this can improve cellular survival as well as promote angiogenesis and metastasis. For example, when macrophages stimulate the CSCs, tumor aggressiveness also increases. This dynamic interplay highlights that intrinsic/endogenous regulation accompanied with extrinsic signals that modulate CSC biology may provide new phenomenal insight to target and affect the function of cancer stem cells [12].

Essential Molecules in the Niche

The maintenance of the balance in the CSC niche depends on the interplay among various components and cells of the microenvironment. Essential molecules that regulate the CSC niche are adhesion molecules, cytokines, and chemokines. Adhesion molecules are proteins on the surface of cells that facilitate cell-to-cell adhesion, binding, and interaction. Stem cell niches have a unique ability to self-renew and connect with other cells, which is crucial for their function. This adhesion is made possible by various molecular pathways and substances present in both stem cells and niche cells. E-cadherin is a well-known cell adhesion molecule that interacts with both intracellular and extracellular parts of a cell thereby modulating cellular adhesion. The interaction of this molecule enhances the binding to proteins associated with cytoskeleton and other cells. Some cadherins can control gene expression and signaling pathways when they bind to specific proteins or receptors on cancer stem cells [13]. For example, N-cadherin in breast cancer stem cells binds to FGFR, leading to activation of PI3K/AKT signaling, promoting stem cell self-renewal and tumor initiation.

Cytokines are small proteins released by cells that act as messengers to coordinate the immune response, inflammation, and cellular communication. They can stimulate or inhibit the activity of various immune cells, such as T cells, B cells, and macrophages. Cytokines are released into the tumor microenvironment by both CSCs and other neoplastic cell types. These cell types include tumor-associated macrophages (TAM), tumor-associated neutrophils (TAN), and myeloid-derived suppressor cells (MDSC) leading to an immunosuppressive environment within tumor stroma. Chemokines are a subgroup of cytokines that specifically direct the movement of immune cells to sites of infection, inflammation, or injury. They act as chemo-attractants, guiding cells to migrate towards the source of the chemokine. SDF-1a is one of the most important chemokines identified together with its receptor CXCR4. CXCR4 plays a vital role in retaining, sustaining and homing of tumor cells on hepatocellular carcinoma (HCC). It is important to note that this receptor is crucial for regulating renewal potential of CSCs. Research has reported that SDF-1 can be produced by mesenchymal stem cells (MSCs), and increased levels of SDF-1 have been associated with metastasis in solid cancers such as breast, liver, or lung cancer. Additional cells inside the niche area that are affected by CSCs are cancer-associated fibroblasts (CAFs). CAFs are a type of cell that plays important role in the development, growth, and progression of cancer CAFs are recognized for their ability to promote cell division, stimulate extracellular matrix (ECM) synthesis, and release critical factors like platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) [14, 15].

Inflammatory components like IL-6, IL-8, TGF-β, NFκ, and TNF-α create a tumor microenvironment that promotes the migration and invasion of cancer stem cells. Inflammatory cytokines upregulate the NFκB pathway in CSCs leading to regulation of gene expressions of epithelial-mesenchymal transition (EMT) factors such as Twist, Snail, and Slug factors or genes. These factors control EMT that is an important event in cancer progression, metastasis, and stemness. This highlights the importance of NFκB in migratory and invasive behaviors of CSCs in diverse human malignancies including pancreatic carcinoma, cutaneous melanoma and ovarian tumors. The inflammatory response in the niche is regulated by a complex network of molecules, some of which also play roles in other pathways and functions [16].

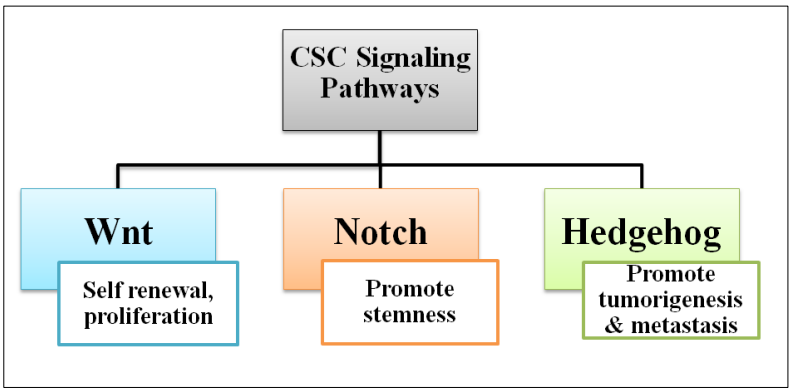

Figure 2: Signaling pathways in Cancer stem cells. Wnt signaling pathways regulate self-renewal and proliferation. Notch and Hedgehog signaling pathways promote stemness and tumorigenesis & metastasis, respectively.

Epigenetic and Genetic Modulations in the CSC Niche

The CSC niche is also influenced by epigenetic modifications, such as DNA methylation and ubiquitination, which interact to regulate CSC behavior and maintenance. DNA methylation is a common epigenetic modification that plays a key role in reprogramming gene expression, with significant implications for CSC biology. For instance, studies have shown that BEX1, regulated by DNA methyltransferase 1 (DNMT1), exhibits varying expression levels in patients with hepatoblastoma, CSC-hepatocellular carcinoma (HCC), and non-CSC HCC. Additionally, activation of the Wnt/β-catenin signaling pathway is essential for maintaining and self-renewing liver CSCs. Furthermore, ubiquitination, a post-translational modification, is vital for CSC maintenance, self-renewal, differentiation, and carcinogenesis. Notably, a recent study demonstrated that targeting MYH9 can inhibit the ubiquitination of GSK3β, leading to the activation of the β-catenin destruction complex. This, in turn, inhibits cancer stemness and EMT in hepatocellular carcinoma, highlighting a potential therapeutic strategy [17].

The genetic diversity of cancer cells has sparked interest in understanding the genetic characteristics and functions of CSCs. Within CSC populations, molecular subgroups can influence their behavior, including their level of dormancy or activity, which in turn affects their ability to survive chemotherapy. The dynamic interaction between CSCs and their microenvironment suggests that changes in the genetic makeup of CSCs or their niches can determine their fate and interactions. This complex relationship allows CSCs to adapt and cycle through different states, enabling them to evade unfavorable changes and thrive in their environment [18].

Implications for CSCs: Opportunities and Challenges

Tailoring cancer therapies to target cancer stem cells can lead to a significant increase in their effectiveness and potentially lead to more durable responses. However, it also comes with substantial risks. The features of CSCs, including their resistance to conventional therapies, and inherent heterogeneity represent challenges in treatment. Present strategies involve using monoclonal antibodies, CAR T-cell therapy, and small-molecule inhibitors to target CSCs selectively. For example, monoclonal antibodies can be produced that selectively target and attach to CSC associated markers and activate the immune system for their elimination. Recently, in cell-based therapy, the treatment that has been made possible through CAR T-cell therapy has been attempted where a patient's T cells have been genetically altered to identify CSC specific antigens, and it has been found effective in the preclinical studies and early phase clinical trials. However, the emergence of resistance mechanisms and the ability of CSCs to respond to therapeutic pressures remain a challenge. Further studies are being conducted to understand changes at the molecular level that possibly make CSCs more resistant or allow them to activate other survival pathways. Furthermore, the isolation of new CSC-specific markers might help in the design of more efficient therapeutic intervention. The challenge lies in developing therapies that can effectively eliminate CSCs without inadvertently creating new populations of resistant cells. Research suggests that CSCs can migrate to other parts of the body, settle, and form new tumors, contributing to cancer metastasis. This highlights the need for a dual treatment approach that targets both the primary tumor and any secondary tumors that have formed, using a combination of invasive (e.g., surgery) and non-invasive (e.g., chemotherapy, radiation) methods [5].

Conclusion and Future Directions

Prospective cancer treatments should focus on targeting cancer stem cells, which are believed to be the root cause of tumor recurrence and resistance to treatment. Due to the developing knowledge of CSC biology, new strategies have been introduced which include the use of combination of conventional therapies together with molecular therapies for elimination of CSCs. For instance, integration of chemotherapy with drugs that work on CSC signaling pathways may increase the therapeutic impact of chemotherapy and minimize the risks of recurrence. The treatment should offer on the specific nature of the tumor. Research is underway to study the molecular mechanisms controlling the CSC behavior and the influence of microenvironment or stem cell niches, which will lead to the development of new therapeutic approaches. Such advanced technologies that include CRISPR gene editing and nanotechnology may also help to further enhance the targeting procedures of CSCs. For example, CRISPR could be used to delete genes that are required for CSC survival, and nanoparticles could be used to deliver chemotherapeutic agents to CSCs selectively or to increase their specificity to decrease side effects. The idea of CSCs in the development and progression of neoplasms will bring a new vision in cancer treatment and help increase the life expectancy of cancer patients in many countries. With more research and the discovery of the intricacies of CSCs, the hope is to translate these findings into clinical applications that not only extend the lives of cancer patients but also improve their overall quality of life.

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RESEARCH ARTICLE

Chemokine expression modulations in primary and metastatıc colorectal cancer

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Abstract

Background: Chemokine are small proteins primarily involved in cell movement, immune response, inflammation and tissue repair. Chemokine also plays an important role in disease conditions including cancer growth. The objective of this study was to analyze expressional modifications in the chemokine in primary and metastatic colorectal cancer by using clinical isolates and *in vivo* model respectively.

Methods: Serum samples from a total of 40 colorectal cancer patients (stage II and III) and healthy controls were collected following the due ethical codes. Circulatory levels of the chemokine (CCL family) were quantified using enzyme-linked immunosorbent assay (ELISA). To investigate metastasis associated expression changes of the chemokine, colorectal cancer animal model and microarray methodologies were exploited.

Results: Increased circulatory levels of CCL2, CCL17 and CCL22 were detected in stage II and III colorectal cancer patients when compared with healthy controls. In contrast, CCL11 levels were substantially low in colorectal cancer patients. As far as rat animal model for colorectal cancer liver metastasis is concerned, only CCL2 levels were considerably high during the early phase of colorectal cancer cells' implantation (Day 03) into the liver. Other chemokines did not demonstrate noticeable de-regulation during the whole period of experimental procedures (up to Day 21).

Conclusion: Differential circulatory levels of chemokine were detected in colorectal cancer patients and healthy controls. Liver metastasis of colorectal cancer cells was accompanied by a marked increase of CCL2 during early phase of settlement. The findings reflected a discrete change in the circulatory and metastasis associated with chemokine profile in colorectal cancer.

Key Words: Colorectal cancer, Chemokine, Liver metastasis, Circulatory levels, Expression changes

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INTRODUCTION

Cancer is known as the leading cause of mortality across the globe in the 21st century. Colorectal cancer (CRC) is the 2nd most prevalent type of cancer in terms of mortality and ranks 3rd in terms of incidence, accounting for 9.6% of all new cancer cases globally in 2022, as based on estimations provided by the International Agency for Research on Cancer (IARC)[1-2]. Expressional changes in genes are important events during cancer onset and progression. Among these, chemokine plays a significant role. They are small sized chemo-attractive cytokines (8-14 kDa) responsible for the migration of epithelial cells, endothelial cells and leukocytes [3]. Chemokine influence tumor cell proliferation, stemness, invasiveness, and stromal processes like angiogenesis and fibrogenesis. They also modulate immune cell localization, activation, and interactions in the tumor microenvironment (TME), thus shaping immune responses [4]. Till now, over 50 chemokine have been identified and classified into four major subgroups known as the CC chemokine, CXC chemokine, CX3C chemokine, and C chemokine depending on the cysteine residues [5]. In this big family, 28 are CC chemokine [6] and among these CCL2, CCL11, CCL17, and CCL22 are under considerations in this study to explore their levels in clinical isolates and a liver metastasis model in rats. CCL2, also called Monocyte Chemoattractant Protein-1 (MCP-1), attracts macrophages and monocytes, thus participating primarily in tumor associated inflammation. It supports the preservation of an immunosuppressive atmosphere, boosts proliferation and motility of cancer cells [7]. CCL11 (Eotaxin) principally recruits eosinophils, playing a role in inflammatory responses. CCL17 is referred as Thymus

and activation-regulated chemokine (TARC) and CCL22, macrophage-derived chemokine (MDC), which controls migration of Th2 and Tregs., regulates the recruitment of these cells, which are often associated with immune suppression in tumor environment [8, 9]. The pro- or anti-cancer role of chemokine is a big debate and based on different types of chemokine, and cells/environment involved, differential response can be expected. Despite their renowned functions in different cancers, circulating levels of the above-mentioned chemokine in CRC patients and metastatic phase require further attention. Studying the variations of these chemokine levels could offer a glimpse at their potential as diagnostic/prognostic biomarkers and/or therapeutic targeting in CRC.

METHODS

Sample Collection

A total of 3-5 mL of blood from naïve CRC patients (22 stage II and 18 stage III) and gender/age matched healthy controls (40) were collected. Blood samples were transferred to BD serum separator vials. After a 30-minute coagulation period at room temperature, specimens were centrifuged at 1000 x g for 15 minutes to separate the serum. The serum was kept in nuclease-free vials at ≤ -80°C for further processing. Details about the participants are provided in Table 1. Informed consent was obtained from all patients and healthy controls. The procedures performed were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

ELISA

Multi-Analyte ELISArray (Qiagen) kit was used to detect the concentrations of chemokine within the serum samples. Briefly, the healthy controls were randomly divided into two groups and pooled separately (20 samples/pool). In similar fashion, stage II and III CRC samples were divided into two groups and pooled (11samples/pool for stage II, 9samples/pool for stage III). 50µl aliquots from every pool were poured into distinct wells of the plate and the concentrations were measured as per manufacturer's guidelines. Absorbance readings were noted at 450nm wavelength utilizing a microplate reader.

Microarray

Microarray analysis was performed to highlight expressional modifications in CCL family of chemokine during the process of CRC liver metastasis. For this purpose, RFP-labelled rat CRC cells (CC531) were transplanted into the rat liver via hepatic portal vein mimicking a liver metastasis model. Afterwards, the growing cells were re-isolated at discrete time intervals (3, 6, 9, 14 and 21 days) followed by RNA extraction and cDNA microarray. A fraction of re-isolated cells was also cultured *in vitro* for 14 and 22 days to compare the results with *in vivo* grown tumor cells.

Data Analysis

Data was entered and analyzed by using SPSS 26.0 (statistical package for social science), where mean values were given for quantitative data. The qualitative variables (gender, age) were expressed as frequencies and percentage. An independent T-test was used to compare numerical variables (chemokine levels) between healthy individuals and CRC patients. A p-value of ≤0.05 was considered as statistically significant.

Table 1: Demographic data of the participants.

	Controls	Stage II CRC	Stage III CRC
Numbers	40	22	18
Gender	Male 26 (65%) Female 14 (35%)	Male 12 (55%) Female 10 (45%)	Male 11 (61%) Female 7 (39%)
Age (years)	Range 26-59 Average 48	Range 28-64 Average 49	Range 29-66 Average 51

RESULTS

Circulatory levels of CCL family of chemokine in colorectal cancer patients

The present work highlighted the circulatory levels of various chemokine ligands present in the serum of CRC patients and were compared with those of healthy controls. In our continuous sampling, most of the samples (>90%) were from stages II and III of CRC, so we restricted ourselves to the patients with these stages. The mean CCL2 level of healthy controls (56pg/ml), was slightly but not significantly lower than those of patients with stage II (62pg/ml) or stage III (74pg/ml) CRC. In contrast to this, the average concentration of CCL11 in healthy controls was higher (122pg/ml) than that of stage II (76pg/ml) and stage III (66pg/ml) CRC patients. The average circulatory level of CCL17 was highest in stage II CRC patients (45pg/ml), whereas that of stage III patients (34pg/ml) was significantly lower ($p < 0.03$), as was that of healthy controls (29pg/ml). CCL22 followed a similar pattern as observed for CCL2, where a gradual, but non-significant increase in circulatory level was found in stage II (75pg/ml) and III (89pg/ml) CRC patients as compared to healthy controls (68pg/ml). Observed changes in levels of the CCL family are shown in Figure 1.

Expression modulations in chemokine during colorectal cancer liver metastasis

Metastasis is a complex mechanism, where cancer cells leave their primary and move to distant secondary sites for further growth. This process is accompanied by numerous expressional changes, which enable the cells for metastasis. To evaluate the potential changes in the CCL chemokine during CRC liver metastasis, CC531 cells (rat CRC) were implanted into rat livers and following re-isolation of the cells after 3, 6, 9, 14 and 21 days, expressional profiling was done via cDNA microarray. The results were compared with the microarray data of the cells re-isolated on day 21 and then cultured *in vitro* for 14 and 22 days. Overall changes in the expression levels of selected chemokine during this period are shown in Figure 2. The majority of CCL chemokine (CCL11, CCL17, CCL22) showed marginal change (≤2fold) during the complete period of experiment. In contrast, CCL2 was the only CCL chemokine with substantial expressional modifications. More specifically, these changes were obvious during the early implantation period (Day 03) and *in vitro* culture (Day 14). To summarize, substantial de-regulation of expression was observed in CCL2 chemokine only during CRC liver metastasis (Figure 2).

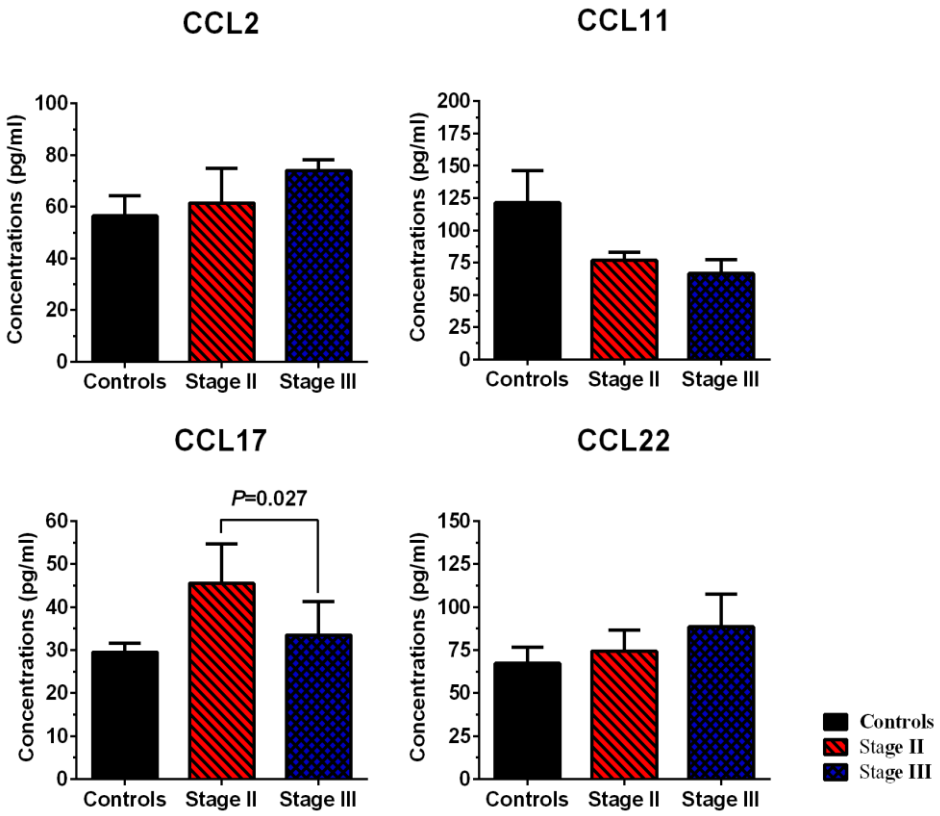

Figure 1: Chemokine concentration among healthy controls, stage II and III CRC patients. Serum samples of the patients were used to determine the levels of selected chemokine while using ELISA methodology.

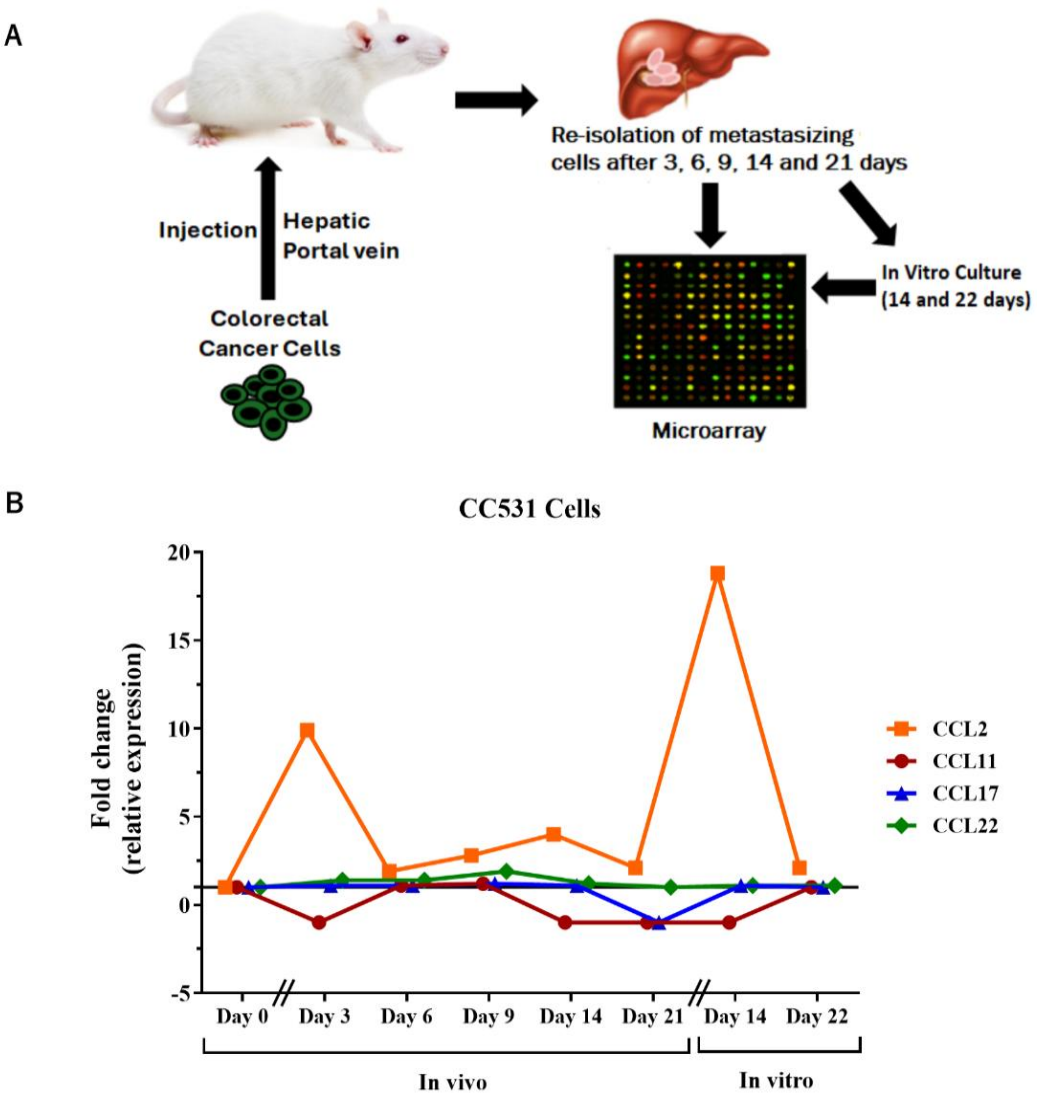

Figure 2: Evaluation of CCL chemokine expressions during colorectal cancer liver metastasis. **A.** Rat colorectal cancer cells were implanted in the liver, re-isolated after selected time intervals and used for expression analysis via cDNA microarray. **B.** Expression modulations in the chemokine' RNA are shown over a period of colorectal cancer liver metastasis, while using *in vivo* model.

DISCUSSION

Differential expressions of the chemokine pathways in disease states is a well-recognized phenomenon. Depending on their kind, concentration, and tissue involvement, chemokine might assist or hinder disease progression. In cancer, this complicated network governs immune cell infiltration, tumor development, survival, and migration. Numerous cells, including immunological, stromal, and tumor cells, express and respond to chemokine variations. Despite substantial research, specific chemokine profiles for certain cancer types remain unknown, particularly in terms of circulatory levels against tissue expression. Given chemokine network's functional involvement in cancer, more investigation is needed regarding its circulating levels. In this study, the focus was to identify the circulatory of CCL chemokine in clinical isolates of colorectal cancer patients. Over 90% of patients in this study had advanced CRC (stage II and III), likely due to late diagnosis, lack of early screening, asymptomatic disease, and/or weak local healthcare structure. To ensure statistically significant results at the end of study, we focused on stage II and III samples. Chemokine levels were measured using an ELISArray plate coated with antibodies for each chemokine, and results were compared with healthy controls. Distinct but mainly non-significant changes in chemokine concentrations were observed in CRC patients compared to healthy controls. CCL2 ligand has been reported to promote carcinogenesis and metastasis of CRC [11-12]. In this study, non-significantly elevated levels of CCL2 were observed in both stage II and III CRC patients. In fact, a gradual increase in CCL2 was observed with the increasing stage of CRC, which shows that higher circulatory levels of CCL2 are related to more advanced stages of the disease and may play a direct role in further progression. CCL11 circulatory levels were distinctly lower in CRC patients in comparison to healthy individuals in this study. CCL11 has been mainly associated with immune cell migration to tumor lesions and plays a pivotal role in metastasis process. A significant decline in CCL11 levels has been associated with decreased immune cell infiltration to tumor area in CRC, which ultimately helps tumor cells to escape from the anti-tumor immunity [13]. This study showed higher expression of CCL17 and CCL22 in CRC patients when compared to the normal healthy controls. In our results, only CCL17 chemokine showed statistically significant differences ($P \leq 0.03$). Other studies showed elevated expressions of CCL17 and CCL22 mRNA expressions in CRC tumors which strengthen the findings of this study. Moreover Bioinformatics analysis showed that high CCL22 expression is linked to increased immune-suppressive cells and decreased antitumor immune cells [14-15]. Metastasis is a complex mechanism that imposes the major challenge while treating cancer. Considering this, precise knowledge about the molecular mechanisms involved in metastasis is crucial. In this study, while using a rat model for CRC liver metastasis, we explored the expression changes in CCL chemokine. CCL2 was the only chemokine among the selected members, which showed a substantial change in expression. These modifications were especially witnessed during early period of implantation in rat liver (Day 03) or *in vitro* culturing (Day 14). This trend shows that higher levels of CCL2 are required by the CRC cells during early settlement period in the new environment. Thus, from therapeutic perspective, targeting the CCL2 during the initial phase of metastasis can be instrumental. As we know, changes in RNA levels ultimately lead to protein level alterations and observed variations in the liver metastasis rat model can be extrapolated to human diseased conditions. In summary, chemokine showed differential level of expression in CRC patients as compared to healthy individuals. As the chemokine levels were detectable in picogram levels, they can be used as prognostic markers especially when their levels are higher in cancerous conditions. Furthermore, discrete changes observed during *in vivo* part of this study show a potential of targeting CCL chemokine (CCL2 for instance) during CRC liver metastasis.

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Ethics Approval: Animal experiments were approved by the relevant governmental animal ethics committee (Regierungspräsidium Karlsruhe, Germany) and all institutional guidelines for the care and use of animals were followed. For clinical investigations, the study was approved by the Ethical Review Committee for Medical and Biomedical Research, University of Health Sciences, Lahore, Pakistan.

Author Contributions: Syeda Faiza collected the clinical samples and performed the ELISA experiments. Afraz Numan analyzed the data and helped with manuscript writing. Martin R. Berger helped *in vivo* experiments. Asim Pervaiz conceived the study, supervised the experiments and wrote the manuscript draft.

Competing Interests: The authors declare that they have no conflict of interest.

Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

RESEARCH ARTICLE

Cytotoxic effects of alkyl-phospholipids (Erufosine and Perifosine) in combination with cell cycle check point inhibitor (ATM) in breast cancer cells

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Abstract

Background: Breast cancer is the most common cancer in females with continuously growing incidence over the last two decades. In advanced stages, the disease is difficult to manage and imposes a major morbidity and mortality burden. Available treatment options are limited with a moderate capacity to cure. Situation demands to look for alternative therapeutic compounds. Alkyl-phospholipids (ALPs) are attractive options, and the two latest generations of this class (erufosine and perifosine) have shown substantial anticancer potential against cancer. In addition, ATM, an important factor during DNA damage/response, has come up for its corresponding therapeutic relevancy. Combining the two above-mentioned classes can lead to effective therapeutic options against breast cancer.

Methods: Toxic effects of the selected compounds on breast cancer cell lines were determined by MTT dye reduction assay. The selected cell lines (MDA-MB-231 and MCF-7) were cultured in 96-well plates and exposed to various concentrations of the compounds for three different time intervals (24, 48 and 72 hours). Following the exposure of cells with the agents, expression modulations in CDKN family of genes were monitored at transcriptome level via real-time PCR methodology.

Results: Exposure with the ALPs and ATM inhibited cell proliferation of the two breast cancer cell lines. Overall, ALPs were more capable of inhibiting cell proliferation. Combination of ALPs and ATM worked synergistically and reduced the cellular proliferation more effectively especially in MCF-7 cells. ALPs and the selected ATM inhibitor induced the expression of CDKN family members. Overall, the induction was maximally seen in CDKN1A gene with a maximum induction level of 40fold in MCF-7 cells.

Conclusion: ALPs and ATM have the potential to inhibit proliferation of breast cancer cells and can support each other synergistically. Important cell cycle inhibitor genes like CDKN family can be induced by using above mentioned compounds.

Keywords: Cancer, Alkyl-phospholipids, Erufosine, Perifosine, ATM, Anti-proliferative, Treatment

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INTRODUCTION

Breast cancer is the term that refers to a disease that affects the mammary glands, that often develops in the cells that line milk ducts and the lobules that feed milk to the ducts [1]. When both genders are considered, breast cancer is the second most prevalent cancer. On physiological and clinical basis, breast cancer is a disorder that has multiple identified histology and molecular subtypes each with its own etiology, risk factors, treatment response and diagnosis [2]. Each year, around 1.5 million of women (that is about 25 percent of all cancer patients) are diagnosed with breast cancer [3]. Multiple types of therapies (i.e., targeted, hormonal, radiation, chemotherapy) along with surgery are some of the treatment options that have been used to treat breast cancer patients. Treatment options are limited, mainly in advanced stages of disease. The heterogeneity of this disease, as well as the present treatment's adverse effects and limited therapeutic choices, are the major hurdles in its management [4].

Cell cycle regulation is done by the presence of specific check-points and restriction points that ensures the integrity of DNA and appropriate cellular environment for a cell to enter in the division phase [5]. These check point pathways are frequently affected in cancerous cells and triggered in response to DNA damage [6]. DNA damage response (DDR) is complex network of mechanisms that sense any damage to DNA and send out signals for further processing in order to keep the genome intact [7]. Defective DDR is one of the hallmarks of cancer, targeting DNA repair pathways have been recognized as a viable area of study for anticancer therapy [8, 9]. Ataxia telangiectasia-

mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) are two core routes through which DDR is initiated. These are protein kinases that have a wide range of physiological functions. They are mainly involved in DNA damage response regulation to survival of cell, its proliferation, cellular metabolism and differentiation [10]. When double standard breaks are made, ATM is a core transducer that phosphorylates multiple effectors in downstream pathway and plays its role in DNA damage repair and autolysis [11], while in case of single-stranded DNA (ssDNA) breaks, ATR is triggered that leads to repair this break [12].

Many chemotherapeutic medicines and radiation have an anti-tumor impact by inducing DNA damage in cancer cells; hence, stimulation of DNA damage response (DDR) pathways is regarded as a significant factor influencing treatment resistance. When some DDR pathways are inactivated, the inhibition of other DDR pathways can induce cancer-specific synthetic lethality. As a result, DDR pathways are seen as attractive options for cancer treatment [13-15]. For cancer treatment ATM and ATR are two promising therapeutic targets due to their functions in regulating responses to DNA mutilation. Chemotherapeutic activity and radiation resistance are both hampered by the activation of ATM signalling [16, 17]. Inhibition of ATM activity is crucial for improving a patient's responsiveness to anti-cancer medication. Many types of cancer have been successfully treated with chemotherapeutic medicines in combination with inhibitors of ATM/ATR or combination of these ATM/ATR inhibitors with radiation therapy.

Synthetic chemicals like alkyl-phospholipids (ALPs) have been shown to have anti-cancer and anti-proliferative properties. Perifosine is a second-generation ALP that has exhibited toxicity against a variety of cancer cell lines. It inhibits many important signal transduction pathways in human malignancies, including PI3K/AKT; which is one of the most important pathway for cancer cell proliferation [18]. Erufosine is a third-generation ALP molecule with 22 carbon additional chain and has been shown to be effective against leukaemia, multiple myeloma, breast, prostate, and squamous carcinoma cell lines. When evaluated for anticancer potential, erufosine has demonstrated considerably reduced off target effects due to structural alteration. *In vivo*, erufosine is more metabolically stable, with lower hemolytic activity and less bone marrow damage. Because of these properties, erufosine can be administered intravenously to obtain clinically meaningful doses that are not achievable with other ALPs [19].

In the present study, we investigated cytotoxic effects imposed by ATM inhibitor and erufosine/perifosine alone and in combinations against breast cancer cells. In addition, expressional analysis of three important genes of CDKN family (CDKN1A, CDKN1B and CDKN2B) were assessed by real time PCR methodology after exposing the cells with the test compounds alone and in combination for comparisons. The findings will be helpful to further evaluate potential utilization of erufosine/perifosine and ATM inhibitor as a part of combinational therapeutic approach to treat breast cancer in future.

METHODS

Cell Cultures

Human breast cancer cell lines (MDA-MB-231 and MCF-7), free of pathogenic contamination, were cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamate, streptomycin (100µg/ml) and penicillin (100IU/ml). Cells were incubated at standard incubation conditions (5% CO_2, 37˚C, humidified environment).

Growth Curves of the Cells

Selected cell lines were cultured in 96-well culture plates (1000-6000 cells/well/100µl complete medium), followed by incubation for three different time points (24, 48 and 72 hours) at standard cell culture incubation conditions. At the end of each point, MTT solution (10mg/ml in PBS) was added (10µl/well) followed by another incubation period of 3 hours. The purple crystals of formazan were formed by viable cells. These crystals were dissolved by adding 50µl/well of DMSO. Optical densities were measured by microplate reader (540/690 nm) and growth curves were generated with time intervals on X-axis and absorbance values on Y-axis.

MTT Dye Reduction Assay (Single Agent Exposure)

MTT dye reduction assay is a colorimetric assay used to assess cell viability and proliferation. To perform this assay, selected breast cancer cell lines (MDA-MB-231 and MCF-7) were seeded at pre-determined cell densities (4000cells/well) in 96-well plates (100µl medium/well). Plates were incubated overnight at standard incubation conditions (5% CO_2, 37˚C, humidified environment) and cells were allowed to grow and attach to the surface of the well. Next day, cells were exposed with various concentrations (1.56-50µM) of erufosine, perifosine and ATM inhibitor (Abcam, Ab219506) for three time points i.e., 24, 48 and 72 hours. Following the exposure intervals, MTT solution was added, and optical densities were determined as explained above. Cell survival rates were calculated as percentage of control (untreated) while inhibitory concentrations (IC) were calculated by GraphPad Prism 10.3 software.

MTT Dye Reduction Assay
(Exposure with Combinations of Compounds)

The cells were seeded in 96-well plates as described above and were treated with combination of ATM inhibitor and erufosine/perifosine as per inhibitory concentrations (IC) generated from above-mentioned experiment (Table 2) for three-time points i.e., 24, 48 and 72 hours. Following the treatment intervals, MTT assay was performed, and viable cell population was determined as explained above. In both cases (single agent or combinational treatment), the assays were performed in triplicate and the results were obtained by at least two independent experiments. Results were compared with untreated control cells growing in parallel.

Expressional Profiling
Treatment with selected compounds

The selected breast cancer cell lines were seeded (200,000cells /2ml complete medium/well) in 6-well plates. Plates were incubated overnight at standard incubation conditions (5% CO_2, 37˚C, humidified environment) and cells were allowed to grow and attach to the surface of the well. Next day, the cells were exposed with various inhibitory concentrations of erufosine, perifosine and ATM alone (IC25 and IC50) and in combinations (IC25+IC25) (Table 3). After 48 hours of incubation, media was discarded from each well, the cells were washed with 500 µl of PBS, trypsinized and cell palettes were collected by centrifugation at 3000rpm for 5 minutes and stored at -80˚C.

Table 1: Treatment of breast cancer cells against different concentrations of selected compounds for MTT assay

Cell Lines	Erufosine (µM)	Perifosine (µM)	ATM (µM)
MDA-MB-231	1.56, 3.12, 6.25, 12.5, 25, 50	1.56, 3.12, 6.25, 12.5, 25, 50	1.56, 3.12, 6.25, 12.5, 25, 50
MCF-7	1.56, 3.12, 6.25, 12.5, 25, 50	1.56, 3.12, 6.25, 12.5, 25, 50	1.56, 3.12, 6.25, 12.5, 25, 50

Table 2: Exposure with inhibitors and compounds in combinations

Cell Lines	Erufosine + ATM		Perifosine + ATM	
	Erufosine	ATM	Perifosine	ATM
MDA-MB-231	IC25	IC25	IC25	IC25
MCF-7	IC25	IC25	IC25	IC25

Table 3: Exposure with single agents and combinations for real-time PCR analysis

Cell Lines	Single Agent Treatment			Combinational Treatment
MDA-MB-231	Erufosine IC25	Perifosine IC25	ATM IC25	Erufosine IC25+ ATM IC25
	Erufosine IC50	Perifosine IC50	ATM IC50	Perifosine IC25+ ATM IC25
MCF-7	Erufosine IC25	Perifosine IC25	ATM IC25	Erufosine IC25+ ATM IC25
	Erufosine IC50	Perifosine IC50	ATM IC50	Perifosine IC25+ ATM IC25

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from the control and treated cells by using commercially available extraction kit (Thermo Fisher Scientific: Cat#K0731). The extracted RNA was quantified and stored at -80°C immediately for further use. A total of 1000ng extracted RNA/samples was used to synthesize cDNA by using the reverse transcriptase kit by Thermofisher scientific (cat # K1622). To verify the prepared cDNA samples, PCR based amplification of a reference gene (HPRT1) was performed. Afterwards, a total of 10µl of the amplified product and was loaded on 2.5% agarose gel and visualized by gel electrophoresis.

Primer Designing and Optimization

Primers of the selected genes (CDKN1A, CDKN1B and CDKN2B) were designed by choosing gene sequence from NCBI Genbank and using Primer3 software. Primer sequences are given in Table 4. The designed primers were optimized by gradient PCR methodology in which three different annealing temperatures were tested (56, 59 & 62°C). The amplified products were analyzed by using gel electrophoresis. The annealing temperature was selected by observing the quality of bands on the gel.

Real Time PCR

Quantitative real time PCRs (qRT-PCRs) were performed for the selected genes (CDKN1A, CDKN1B and CDKN2B) by using SybrGreen florescence dye (Thermo Fisher Scientific, Cat#K0221), prepared cDNA samples from the two breast cancer cell lines treated with different concentrations of the compounds alone or in combination along with gene specific primers. All the samples were amplified in triplicate, while Quantstudio3™ machine was used for real-time PCR. For normalization of qRT-PCRs data, the expression levels of (HPRT1) gene were used as reference and the fold changes of the selected genes were calculated by the Livak (2-ΔΔCT) method.

Data Analysis

Cytotoxicity data generated from MTT assay was presented as frequency percentages. For real-time PCR analysis, fold changes were calculated by Livak $2^{-\Delta\Delta CT}$ method by comparing Cq values of treated and untreated control samples.

Table 4: Primer sequence for amplification of selected genes

Gene	Forward	Reverse
CDKN1A	GCTTCATGCCAGCTACTTCC	CTGTGCTCACTTCAGGGTCA
CDKN1B	CCGGCTAACTCTGAGGACAC	TGCAGGTCGCTTCCTTATTC
CDKN2B	GACCGGGAATAACCTTCCAT	AAACCCTGAAAAGCAAACGA
HPRT1	GACCAGTCAACAGGGGACAT	CTTGCGACCTTGACCATCTT

RESULTS

Growth Curve

MTT dye reduction assay was done to optimize the cell densities for subsequent experiments. For this purpose, the cells were cultured in 96-well culture plates (1000-6000 cells/well/100µl medium) for 24, 48 and 72 hours at standard cell culture incubation conditions. After adding DMSO, formazan crystals produced purple color of different shades, showed the presence of viable cells. Optical densities were determined by an ELISA plate reader (540/690nm) and growth curves were generated with number of cells on X-axis and absorbance values on Y-axis shown in Figure 1. Continuous proliferation of breast cancer cell lines (MDA-MB-231 and MCF-7) was observed for 4000cells/well for all three-time intervals, while for further higher numbers (5000 and 6000 cells/well), there was a clear reduction in proliferation especially for 72 hours. This reduction in later time intervals was likely due to overabundance of cell population having limited space for attachment and further growth. In addition, lack of nutrients and production of waste could be additional limiting factors in case of these higher cell numbers/well.

Cytotoxic Effects of Erufosine, Perifosine, ATM inhibitor in breast cancer cell lines

Erufosine, perifosine and ATM inhibitor induced substantial anti-proliferative effects in MDA-MB-231 and MCF-7 cells (Figure 2). The most prominent cytotoxic effects were observed in MCF-7 cells; this in turn shows that triple positive breast cancer cells are more prone towards exposure of these compounds. The effects were almost time dependent as the more inhibition of cell viability was observed for a longer period of exposure with the compounds. Additionally, the effects were concentrations dependent (beyond 3.10µM) as more inhibition of cellular growth was witnessed with increasing concentrations. Overall, the effects in response to erufosine and perifosine were comparable, while ATM inhibitor induced fractionally low effects in comparison to ALPs.

Cytotoxic Effects of Erufosine, Perifosine, ATM inhibitor in combinations

As far as effects of the combinations are concerned, almost no synergistic impact of growth inhibition was observed while combining the two alkyl-phospholipids (erufosine and perifosine) in combination with ATM in MDA-MB-231 cells. However, fractional synergism was observed for ATM and erufosine combination only for 24 hours, which shows a short-term better control when the two compounds are combined. A prominent

synergism was observed in MCF-7 cells as compared to MDA-MB-231 cells. Synergistic effects of ALPs in combination with ATM were continuous in MCF7 cells for the three-time intervals. This indicates that combination of erufosine and perifosine with ATM inhibitor is more effective against triple positive breast cancer cells (Figure 3).

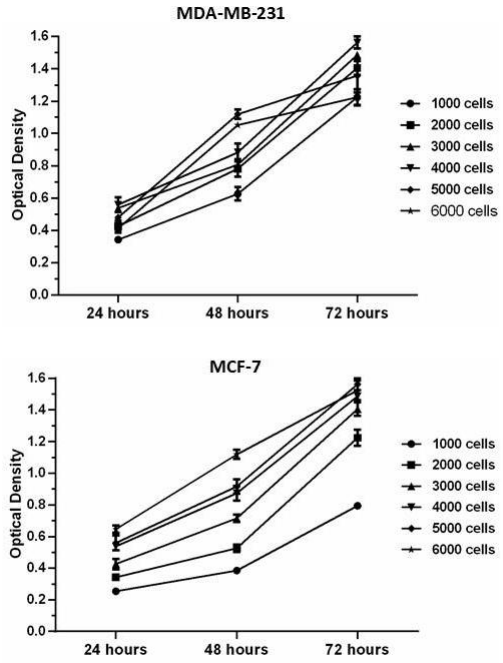

Figure1: Growth curve generation for three different time points

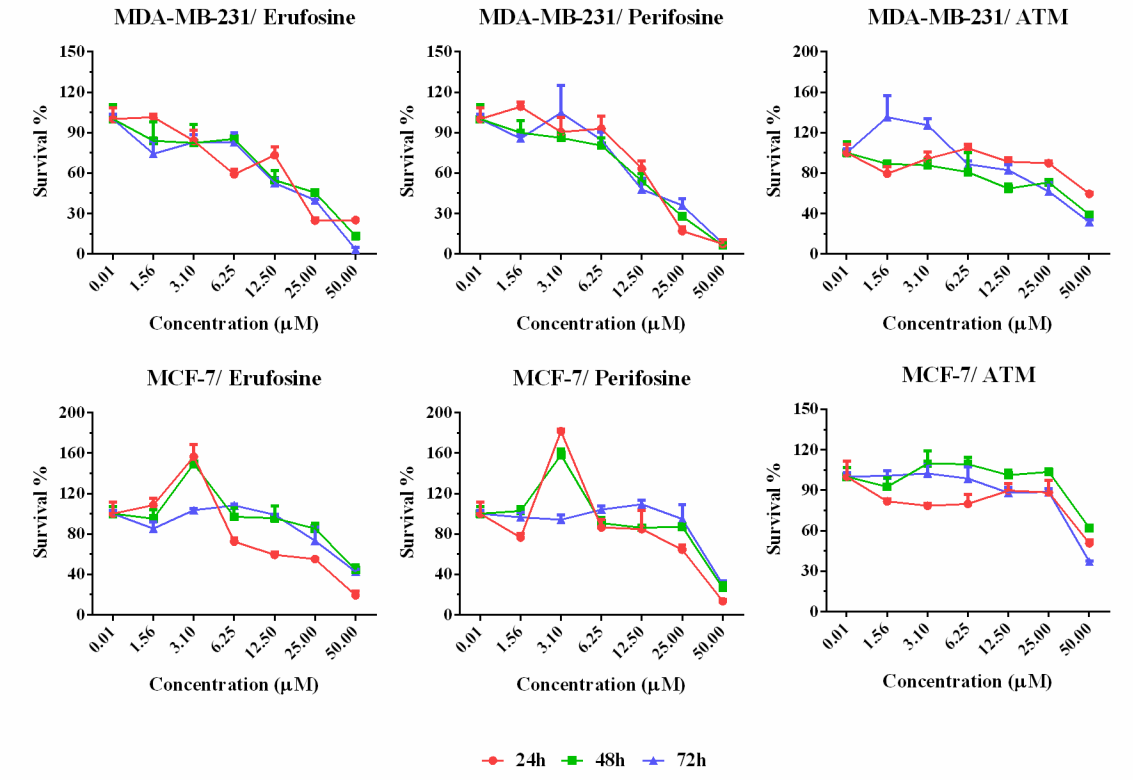

Figure 2: MTT results of breast cancer cell lines (MDA-MB-231 and MCF-7) after treatment with selected compounds (erufosine, perifosine, ATM inhibitor)

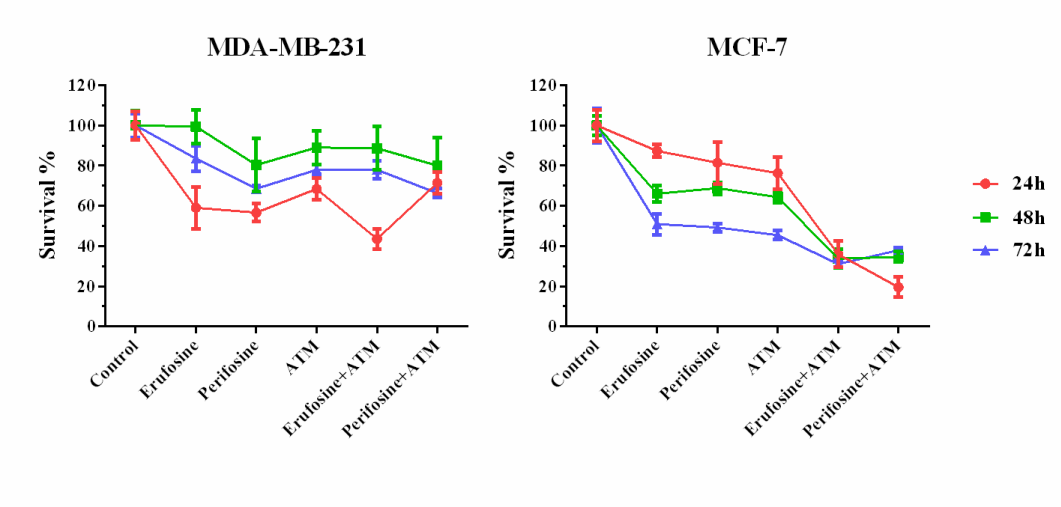

Figure 3: MTT results of breast cancer cell lines (MDA-MB-231 and MCF-7) after treatment with selected compounds (erufosine, perifosine, ATM inhibitor) alone and in combinations

Expressional Analysis of CDKN1A and CDKN1B Genes in MDA-MB-231 cells

A concentration dependent induction of CDKN1A gene was observed in MDA-MB-231 breast cancer cell line after exposure with selected ALPs and ATM inhibitor. An induction of 10.5, 13.5 and 10.3fold was observed in response to IC50 for erufosine, perifosine and ATM inhibitor respectively. The induction of CDKN1B gene was inversely proportional to the applied concentrations for erufosine i.e., maximum induction (2fold) at low concentration (IC25) while declining slightly (1.6fold) at high concentration (IC50). Perifosine and ATM inhibitor induced expression of CDKN1B gene in dose dependent format. The two compounds induced minimal effects at lower concentration (IC25) as shown by maximum induction of 1.9fold. At higher concentration (IC50), perifosine and ATM inhibitor induced the expression by 3.2 and 2.8fold respectively. The combination of erufosine (IC25) with ATM inhibitor induced 1.9fold expression, while combination of perifosine (IC25) with ATM (IC25) inhibitor induced relatively more effective expression of almost 3.4fold in CDKN1A gene. Overall, reduced modulation by combinational approach was observed in CDKN1B gene as compared to CDKN1A gene in MDA-MB-231breast cancer cell line (Figure 4).

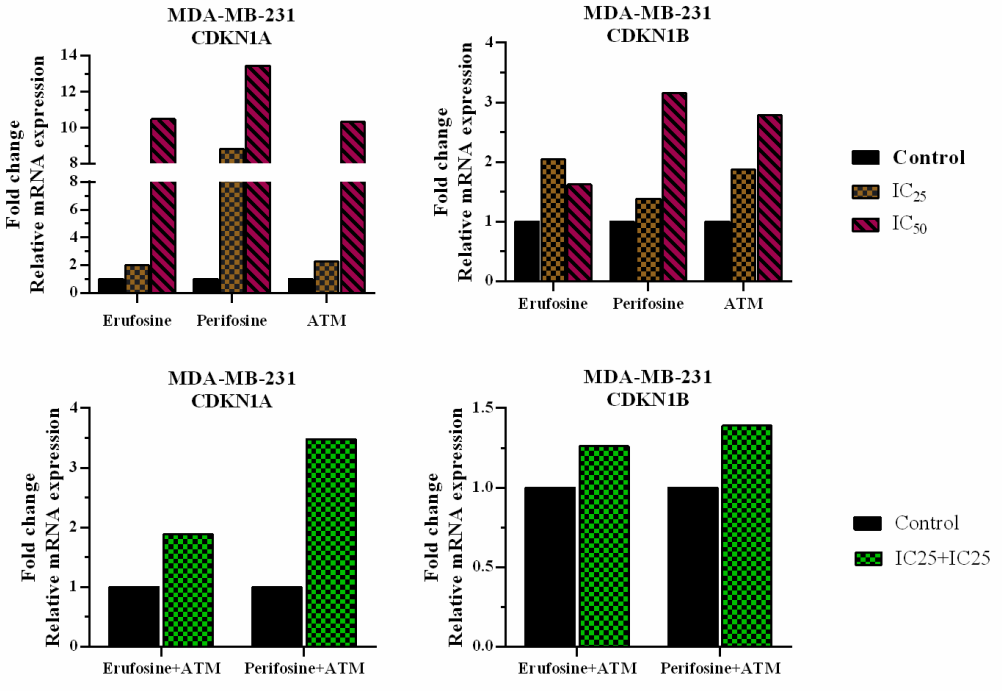

Figure 4: Expressional changes in CDKN1A and CDKN1B gene in MDA-MB-231 cells

Expressional Analysis of CDKN1A, CDKN1B and CDKN2B Genes in MCF-7 cells

Exposure with the selected compounds induced more substantial expressional modulations in MCF-7 cells as compared to MDA-MB-231 cells. In MCF-7 cell line, the gradual decline in induction of CDKN1A gene was observed with increasing ICs of erufosine. Precisely, maximum induction (41.4fold) was observed at IC25 while lowest induction (12.1fold) was observed at IC50 exposure. In response to perifosine, the expression was dose dependent as the effects intensified (6.9 and 9.9fold) with increasing concentration (IC25 and IC50 respectively). The CDKN1A expression in response to ATM inhibitor is quite similar with erufosine. Maximum activation (31.3fold) was observed with IC25, while reduced induction (7.8fold) was witnessed with higher concentration (IC50). Overall, it can be claimed that the compounds altered the expression levels of CDKN1A more effectively in MCF-7 cells.

Almost a uniform response in expressional pattern of CDKN1B gene was observed when MCF-7 cells were exposed to the selected ALPs i.e., erufosine and perifosine. The maximum effect (1.9fold) was observed at IC25 concentration of erufosine and beyond this the effect was diluted as shown by lower expression level (1.1fold). Perifosine induced negligible change in expression levels of CDKN1B gene at both tested concentrations when compared to the untreated control cells. The pattern of expression of CDKN1B gene in response to ATM inhibitor was quite like that of erufosine. Maximum induction was 3.5fold at IC25 while it declined to 1.6fold at higher concentration IC50. All in all, CDKN1B gene expression was minimally affected in response to tested compounds.

A different expressional pattern of CDKN2B gene was observed when MCF-7 cells were exposed to the selected ALPs and ATM inhibitors as compared to the results of CDKN1A and CDKN1B genes. Erufosine induced a negligible change in the expression level of CDKN2B gene at lower concentration (IC25) when compared to the untreated control cells. In contrast, a substantial inhibition of -2.7fold was observed at higher concentration (IC50). Perifosine and ATM inhibitor inhibited expression of CDKN2B gene in dose dependent format. To be precise, there was a moderate inhibition at lower concentration (IC25) while the maximum inhibition of -4.1 and -3.5fold were observed at higher concentration (IC50).

Combination of selected ALPs with ATM inhibitor induced relatively effective alterations in CDKN1A gene in MCF-7 cells as compared to MDA-MB-231 cells. The maximum observed induction was 8.7fold when exposing the cells to the combination of erufosine (IC25) with ATM (IC25) inhibitor. Overall, a less effective modulation by combinational approach was observed in CDKN1B gene as compared to CDKN1A gene in both breast cancer cell lines. In MCF-7 cell line, the expression of the CDKN2B gene was inhibited at all tested combinations of erufosine and perifosine with ATM inhibitor. Overall modifications in the genes in response to tested compounds in MCF-7 cells are shown in Figure 5.

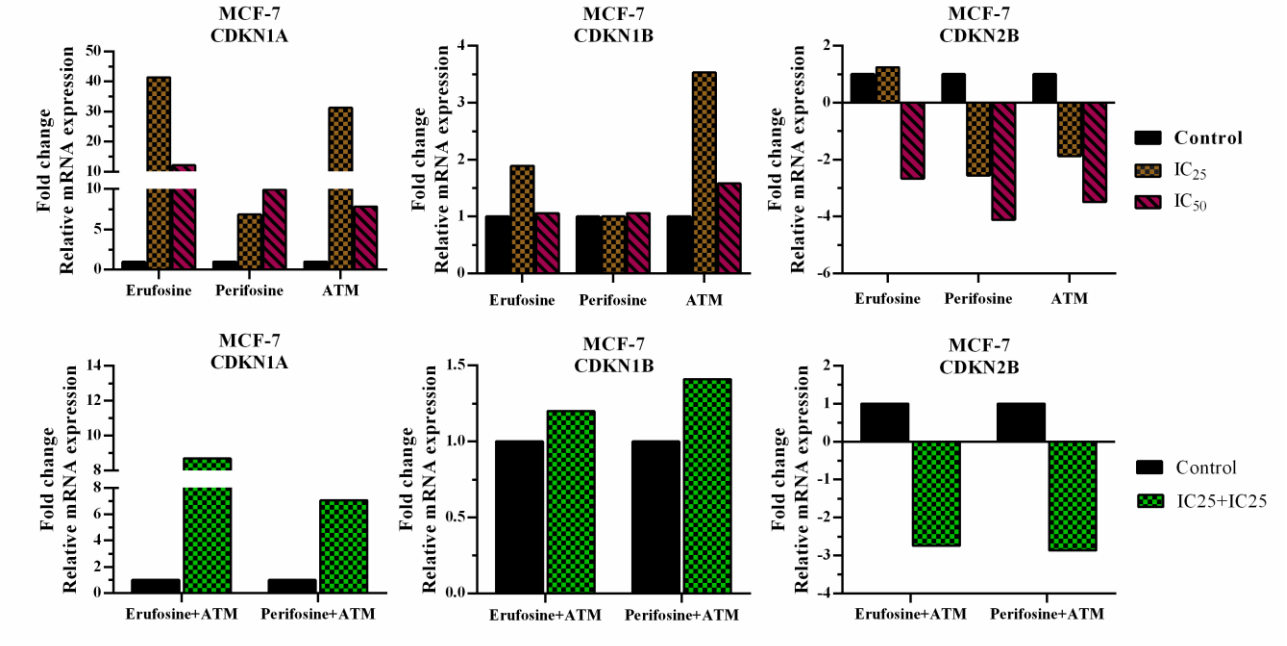

Figure 5: Expressional changes in CDKN1A, CDKN1B and CDKN2B gene in MCF-7 cells

DISSCUSION

Despite significant scientific achievements, breast cancer remains the most frequent cancer in women globally and is a serious public health issue. Female breast cancer is becoming more common every year [20]. According to literature, early screening and detection of breast cancer combined with proper treatment could greatly decrease death rates due to breast cancer. The heterogeneity of this disease, as well as the present treatment's adverse effects and limited therapeutic choices, are the major hurdles in its management [4, 21]. To enhance the present therapeutic regimens, more effective solutions are urgently needed. Immunotherapy, gene therapy, and combinational treatment approaches are among the strategies being researched to overcome the hurdles [22].

Many chemotherapeutic medicines and radiation have an anti-tumor impact by inducing DNA damage in cancer cells. Combinational approach of chemotherapy for effective anticancer effects is a well-accepted fact as it provides an opportunity to control the disease burden by using lower dosages of the compounds with lesser side-effects. For cancer treatment, ATM and ATR are the two promising therapeutic targets due to their vital cellular functions and facilitators in regulating responses to DNA changes. Inhibition of ATM activity is crucial for improving a patient's responsiveness to anti-cancer medication. Many types of cancer have been successfully treated with chemotherapy in combination with inhibitors of ATM/ATR [14, 16, 17]. All is all, ATM and ATR are effective therapeutic targets, and their corresponding inhibitors are being investigated intensively.

ALPs, targeting cell membrane phospholipids, have anti-cancer and anti-proliferative properties against various cancers. Perifosine, a 2nd-generation ALP, inhibits key signal transduction pathways, including PI3K/AKT, and shows clinical effectiveness in combination treatments [18, 23]. Erufosine (3rd-generation ALP), is metabolically more stable, with lower hemolytic activity and less bone marrow damage, able to penetrate the blood-brain barrier and can be administered intravenously to obtain clinically meaningful doses that are not achievable with other ALPs [19]. Combining synthetic ALPs with ATM/ATR inhibitors is considered an attractive area of research.

In this study, we investigated the cytotoxic effects imposed by ATM inhibitor and erufosine/perifosine alone and in combinations in breast cancer cells. For this purpose, two BC cell lines (MDA-MB-231 and MCF-7) were selected and grown in 96-well plates in cell culture settings and exposed to various concentrations of erufosine, perifosine and ATM inhibitor alone or in combinations, followed by assessment of proliferation by MTT dye reduction assay as shown in Table 1 and 2. Based on the results obtained, it is clear that ALPs are more effective in inhibiting the proliferation of breast cancer cells as compared to ATM inhibitor. One of the potential reasons behind this phenomenon could be that ALPs interact with cellular lipids and affect multiple intracellular downstream signaling cascades. In comparison, ATM inhibitors specifically interact with their targets and induce limited overall reaction. Furthermore, development of resistance mechanisms or negative feedback loops in response to ATM inhibitor exposure cannot be figured out. As far as combinations are concerned, the approach was more effective against triple positive breast cancer cells (MCF-7) as compared to triple negative cells (MDA-MB-231). Other than receptor status, further cellular differences among the two cell lines need to be considered before just focusing on molecular subtype dependent response of breast cancer cells.

As we know, genes are the basic moderators of cellular functions, thus, to figure out mechanistic reasoning behind the observed anti-proliferative effects, a key cell cycle related gene family (CDKN) was taken up in this study. This family is comprised of four major cell cycle inhibitors which play a crucial role in cell cycle. Their expression levels can affect cell proliferation and divisions efficiently. As explained in methods, breast cancer cells were exposed to ALPs and ATM in single or combinations and expression levels of four CDKN family members (CDKN1A, CDKN2A, CDKN1B, CDKN2B) were identified via real- time PCR methodology. During the primer optimization step, CDKN2B expression was only seen in MCF-7 cells while CDKN2A did not show any expression in both selected breast cancer cell lines. As per available literature, the two members did not express in all breast cancer cell lines. Furthermore, expression of transcript variant is also possible, against which our designed primer was not compatible. To figure out the exact situation, primers against all known transcript variants of CDKN2A and CDKN2B need to be designed and check for potential expression of these two genes in the selected breast cancer cell lines.

For other two members (CDKN1A and CDKN1B), a clear expression was noticed in both breast cancer cell lines. In response to ALPs, a substantial expression (up to 14fold) was noticed in MDA-MB-231 cells. The effects were more pronounced in MCF-7 cells as a maximum of ~40fold induction was observed in response to ALPs exposure. Interestingly, the results were in line with MTT data where MCF-7 cells were more sensitive towards ALPs exposure. In comparison, the ATM inhibitor was more effective and induced a maximum of ~30fold induction in MCF-7 cells. As far as CDKN1B gene is concerned, overall, a moderate induction (up to 4fold) was observed in both cell lines. Interestingly, the ATM inhibitor was more influencing in this condition in comparison to ALPs. With combinations of ALPs and the ATM inhibitor at lower concentrations (IC25), a substantial induction of CDKN1A and CDKN1B gene was observed in breast cancer cell lines, particularly MCF-7 cells. This in turn indicates that by using lower dosage

of the ALPs and ATM inhibitor, a substantial induction of a vital cell cycle inhibitor (CDKN1A) is possible in breast cancer cells, and this is a valuable hope from clinical perspective. All in all, data suggests that combinations of ALPs and the ATM inhibitor can work synergistically to impose anti-proliferative effects. With further studies to support these findings, ALPs and ATM inhibitors can be a promising combinational therapy to control breast cancer overtime. In this context, existing FDA approved ALPs (Perifosine) can be considered immediately, while an improved ALP (Erufosine) is a promising futuristic compound.

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Data Availability Statement: The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

RESEARCH ARTICLE

Effects of synthetic alkyl-phospholipid (Perifosine) on survival and cell cycle relevant genes of hepatocellular carcinoma cells

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Abstract

Background: Hepatocellular carcinoma (HCC) is a leading cause of cancer-related mortality globally, particularly in Asia. Limited treatment options highlight the needs for novel therapies. Alkyl-phospholipids (ALPs) offer a promising alternative to traditional chemotherapy by targeting cell membrane integrity and signaling pathways rather than DNA replication. Perifosine, a synthetic ALP, has shown cytotoxic effects in cancer cells, but its mechanisms on cell cycle regulation in HCC require further investigation.

Methods: This *in vitro* study evaluated the effects of perifosine on HepG2 liver cancer cells. Cytotoxicity of perifosine was assessed using the MTT assay while applying various concentrations over 24-, 48-, and 72-hours intervals. Gene expression analysis of cell cycle regulator genes (CCNA1, CCNA2, and CCND1) was conducted using qRT-PCR after treating the HepG2 cancer cells with perifosine at IC25, IC50, and IC75 concentrations for 48 hours.

Results: Perifosine demonstrated time and dose dependent cytotoxicity, with increased cell death at higher concentrations and prolonged exposure. Perifosine exposure imposed discrete expression modifications in CCNA1, CCNA2 and CCNDI genes. Maximum de-regulation was observed for CNNA1 (8fold) and CCNA2 (-3.5fold) in response to perifosine exposure.

Conclusion: Perifosine exhibited cytotoxic effects and imposed de-regulation in cell cycle related genes in liver cancer cells.

Keywords: Hepatocellular carcinoma, Perifosine, Cytotoxic, Cell cycle, Cyclins

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a major global concern and one of the major contributors to cancer-related deaths across the globe with limited treatment options. It is the most prevalent liver cancer subtype and represents 75% of all cases of liver cancer. According to GLOBOCAN estimates, liver cancer is listed as the 6th most prevalent type of cancer and is the 3rd most common cause of cancer mortality [1]. In 2020, 72.5% of all the reported liver cancer cases and 73.3% of liver cancer mortality were reported from Asian countries [2]. HCC are typically treated with chemotherapy and immunotherapy. Synthetic ALPs present a promising alternative, potentially offering targeted treatment with reduced toxicity and side effects [3].

Cancer is a complex disorder characterized by many genetic and molecular changes that promote unchecked cell proliferation. Dysregulation of the cell cycle, a strictly controlled precise mechanism that ensures normal cell division and maintain genetic integrity across cellular generations, is an important hallmark of cancer [4,5]. There are many factors which regulate the cell cycle, but two important key regulators are cyclins and cyclin-dependent kinases (CDKs), which control the cell cycle processes through its different phases. Tumor proliferation is often caused by disruptions of the cell cycle regulators, such as loss of CDK inhibitors or overexpression of cyclins or CDKs [6]. In cancer, the regulatory network can get disturbed, thus allowing abnormal cell division to take place. For example, overexpression of cyclins or CDKs might promote cell cycle advancement despite the lack of stimuli for cell growth. Conversely, failure in expression of CDK inhibitors might interfere with key checkpoints, allowing cancer cells to continue replicating [7].

Given the central role of the cell cycle in cancer development, targeted therapeutic strategies are being developed to exploit these dysregulated pathways. Among these strategies are the alkyl-phospholipids (ALPs). It is a novel class of anticancer drugs which target the cell cycle. ALPs are synthetic substances that structurally resemble natural phospholipids but possess some alterations that give their anticancer qualities.

Structural modifications in various members of ALPs have generated new compounds with reduced side-effects and high specificity overtime. Contrary to conventional chemotherapeutic drugs, which mainly target mitotic spindle assembly or DNA replication, ALPs work by interfering with signaling networks and cell membrane integrity and cellular processes like apoptosis, cell cycle regulation and signal transduction pathways [8]. This membrane-targeted mode of action is especially interesting since it gets around some of the typical defense's tumors build up against DNA-damaging agents. Perifosine belongs to 2nd generation of ALPs that have been produced over time and has unique structural characteristics and therapeutic applications [9].

The mechanistic hypotheses for ALPs share a common factor: their presence in the cell membrane. One hypothesis posits that ALPs alter membrane particulate size and distribution [10]. Another assumption suggests that ALPs cause biological and physical disruptions, obstructing phospholipid metabolism, cell division, and associated signaling, while promoting apoptosis through various pathways [11]. Additionally, ALPs may integrate into the membrane and undergo endocytosis via lipid rafts with the help of the ATP-dependent enzyme Flippase, contributing to cell lysis [12]. Perifosine works by targeting the pleckstrin homology domain of Akt, blocking its membrane translocation however clinical validation is still needed to confirm this hypothesis [13].

Among the cell cycle associated genes, there are various members with discrete functions. CCNA1 (Cyclin A1) is involved in controlling the G1 and G2 checkpoints by associating with CDK2 and CDC2 kinases, thus facilitating the progression through these phases. CCNA2 (Cyclin A2) activate CDK2 kinase and promotes the advancement of the cell cycle, which drives the progression from the G1 phase to S phase and from G2 to M phase. Cyclin D1, or CCND1, interacts with the Rb protein to control the progression of the cell cycle and is necessary for the transition from the G1 phase to S phase [14]. Alterations in either expression or structure of CCND1 are commonly observed in various neoplasia, highlighting its role in cancer development. Studying the effects of synthetic ALPs on hepatocellular carcinoma cells, especially on cell cycle-relevant genes, is of vital significance. The present study was designed to understand the potential effects of perifosine in HCC cells and corresponding implications on cell cycle related genes.

METHODS

Cell Culture and Perifosine

HepG2 (a human liver cancer cell line) cells, obtained from Center of Excellence in Molecular Biology (CEMB), Punjab University, were grown in DMEM medium containing 10% fetal bovine serum (FBS), 2mM L-glutamine, and antibiotics (100 µg/ml streptomycin, 100 IU/ml penicillin) to inhibit microbes. In 5% CO2 atmosphere cells were incubated at 37°C passaged biweekly to ensure logarithmic growth. An alkyl-phospholipid "Perifosine" was selected for the study and was purchased from Sigma Company (Sigma Aldrich, Cat#SML0612).

Cytotoxicity of Perifosine

To assess the toxic effects of this compound, the MTT dye reduction assay was performed. In 96-well plates HepG2 cells were seeded at 3000 cells per well and were treated with perifosine at concentrations ranging from 0.78-50 µM, and incubated for 24, 48, and 72 hours. After each interval, the MTT solution poured into each well and incubation of 3 hours was given. To resolve formazan crystals, 50µl of DMSO was add to each well then optical density was recorded at 560/690 nm wavelength via ELISA reader. The inhibitory concentration (IC) was calculated with GraphPad Prism 6 software.

RNA Extraction and Reverse Transcription

In 6-well plates, HepG2 cells were seeded maintaining 150,000 cells per well for expressional analysis, each plate was treated with IC25, IC50, and IC75 concentrations of perifosine for 48 hours, while the IC values were calculated based on MTT assay results. Utilizing the RNeasy Mini Kit (Qiagen, Cat# 74104), RNA was extracted, and its concentration along with purity were measured using Nanodrop ND2000 spectrophotometer. Complementary DNA (cDNA) was prepared via RevertAid cDNA Synthesis Kit (Thermo-Scientific, Cat#K1622) using final 200ng of RNA with the following incubation conditions, annealing at 42°C for 60 minutes, extension at 72°C for 5 minutes. For validation of cDNA synthesis, PCR amplification was performed using GAPDH.

Primer Designing and Real Time PCR

Primers for the target genes were designed using the sequences from NCBI database using Primer3Plus software. The sequences of the CCNA1 primers were 5′-TAGACACCGGCACACTCAAG-3 (forward stand), 5′-CTACCAGCATAGGGGAAACTGT-3 (reverse stand), with 312 bp amplicon size. For CCNA2, the sequences were 5′-CCTCCTTGGAAAGCAAACAG-3′ (forward stand), and the reverse was 5′-TCAAGAGGGACCAATGGTTT-3′ (reverse stand), with 173 bp product size. For CCND1, primer sequences were 5′-GGGGGCGTAGCATCATAGTA-3′(forward), and 5′-

GTGGTGGCACGTAAGACACA-3′ (reverse stand), with 114 bp amplicon size. Expressional analysis was done via real-time PCR using SYBR Green qPCR Master and cDNA templates. The data was normalized using housekeeping gene (HPRT). Livak method (2^ΔΔCT) was employed to determine the relative expression of genes.

RESULTS

Perifosine Mediated Cytotoxic Effects in Liver Cancer Cells

HepG2 cells were exposed to varying concentrations (0.75-50µM) of perifosine over 24, 48, and 72 hours. Cytotoxic effects were evaluated using the MTT assay, with results analyzed via GraphPad Prism 6. The compound exhibited concentration- and time-dependent cytotoxicity, with increasing concentrations leading to greater toxicity (Figure 1). Furthermore, the effects were more prominent over longer time intervals (48 and 72 hours) as there were greater inhibitory effects on the similar concentrations applied. Thus, IC values decreased over time, indicating enhanced cytotoxicity with prolonged exposure. Perifosine was effective in reducing the number of viable cells as shown by a steep decline in the curve. IC values obtained after the selected time intervals are shown in Table 1.

Expressional Analysis of Cell Cycle Genes

Expression levels of the three selected genes i.e., CCNA1, CCNA2 and CCND1 were identified in HepG2 cells by real-time PCR methodology. For this purpose, the cells were exposed to perifosine followed by cDNA synthesis. After normalization of the data, fold changes were identified by $2^{\Delta\Delta CT}$ method to compare the experimental (treated) and control (untreated) groups. In response to perifosine exposure, effects were not concentration dependent. For the initial low concentrations of perifosine (IC25), there was a slight inhibition (-1.5fold) of CCNA1 expression. In contrast, for higher concentrations of perifosine i.e., IC50 and IC75, substantial induction of CCNA1 expression was observed as shown by 4.7 and 8.6fold increase at mRNA levels (Figure 2). A uniform response in expressional pattern of CCNA2 gene was observed when HepG2 cells were exposed to the ALP. Selected compound induced negligible change in expressional levels of CCNA2 gene at lower concentrations (IC25) when compared to untreated controls cells. In contrast, substantial inhibition was observed at higher concentrations of the compounds as shown in Figure 2. More specifically, perifosine inhibited the expression of CCNA2 gene by -1.3 and -3.4fold when the cells were exposed to IC_{50} and IC_{75} concentrations.

Perifosine induced the expression of CCND1 gene in HepG2 cells in a concentration dependent format. A uniform response in expressional pattern of CCND1 gene was observed when HepG2 cells were exposed to perifosine. Tested compound induced minimal changes in expressional levels of CCND1 gene at lower concentrations (IC25) as shown by 1.2fold induction. Higher concentrations of tested compound substantially induced CCND1 gene expression in the target cells as shown by a maximum 2.5fold induction in response to the exposure. Interestingly, there were almost no differences when fold changes were compared for IC50 and IC75 concentrations of the tested compound. This, in turn, shows a plateau stage where HepG2 cells did not show further response to the test compound as far as induction of CCND1 gene is concerned. All in all, perifosine induced discrete expression alterations in HepG2 cells.

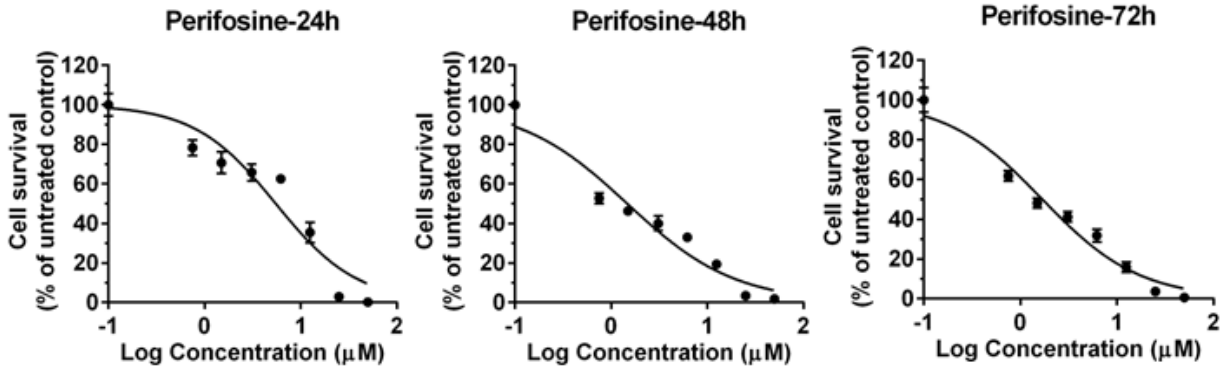

Figure 1: Perifosine induced substantial toxic effects in HepG2 cells as shown by a decline in growth response curves. The effects were calculated by using MTT assay and results were analyzed by using GrapPad Prism v.6 software.

Table 1: IC values of Perifosine against liver cancer cells (HepG2).

Perifosine	IC25	IC50	IC75
24 h	1.9 µM	5.5 µM	16 µM
48 h	0.4 µM	1.5 µM	6.0 µM
72 h	0.5 µM	1.7 µM	6.0 µM

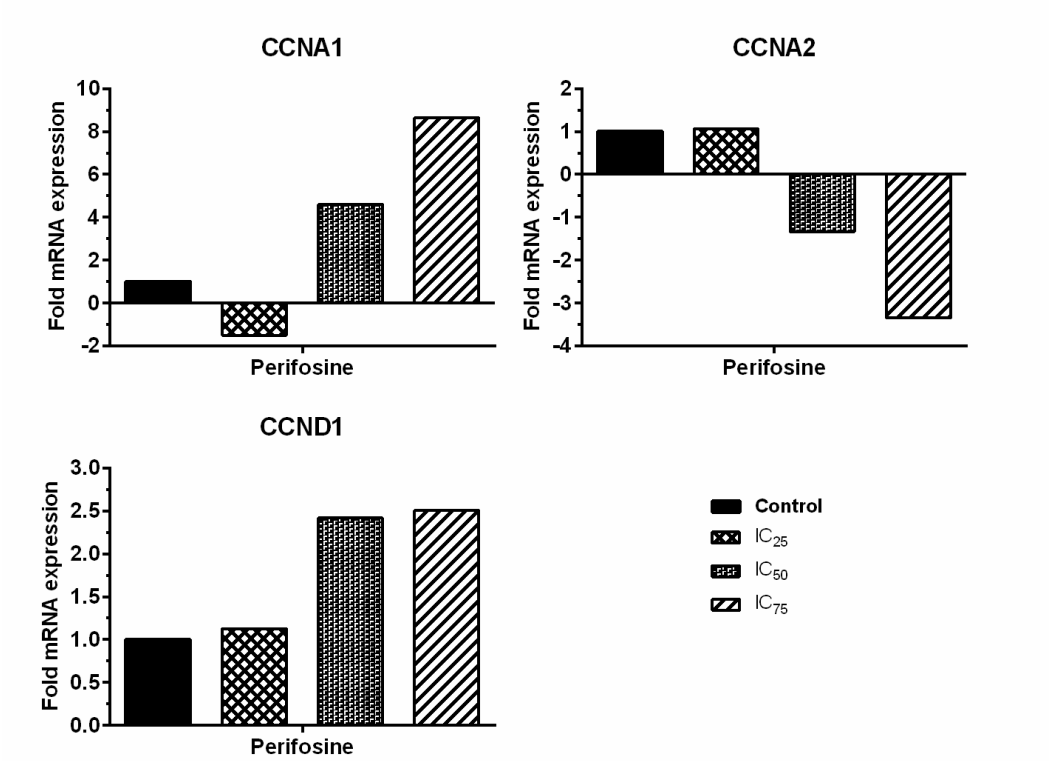

Figure 2: Expressional changes in cell cycle related genes in response to exposure with Perifosine. Exposure with the selected compound de-regulates the expression of genes in discrete fashion as determined by real-time PCR analysis.

DISCUSSION

ALPs have been utilized therapeutically since the mid-twentieth century, with continual structural changes to improve function. This study looked at cytostatic and cytotoxic effects of perifosine in HepG2 cells derived from hepatocellular carcinoma. The exact mechanism through which ALP's are cytotoxic for cancer cells is not clear yet but ALPs are known for blocking for phosphocholine in multiplying cells [15]. The current study was designed to demonstrate insights into the potential effects of perifosine on functional and molecular aspects of liver cancer cells (HepG2).

Perifosine, a 2nd generation ALP, has been shown to suppresses cell survival and trigger apoptosis in neoplastic lung cells by disrupting mTOR signaling and promoting autophagy [16]. Perifosine has also shown cytotoxic effects in cancer cell lines such as HepG2 causing cell cycle arrest and P21 upregulation, triggering apoptosis via caspase and JNK pathways [17]. Cytotoxic effects of perifosine were highlighted by using MTT assay strategy. Following the exposure with compound, substantial inhibition of proliferation was induced. Our results showed that the drug concentration required to inhibit cell growth (IC values) were higher for shorter incubation periods (24h vs. 48h and 72h). Similar kind of growth inhibitory effects of the test compound. For instance, perifosine treatment led to a decrease in hepatoma cell proliferation in a time and dose dependent approach, as measured by the MTT assay [18].

Cancer cells possess multiple genetic defects, which is consistent with the two-hit hypothesis. These cells can be targeted by various mechanisms, as their growth relies on cell division governed by the cell cycle. Because cell growth depends on cell division controlled by the cell cycle, checkpoints in the cycle generally stop mitosis when errors such as DNA damage occur. However, these checkpoints are impaired in cancer cells and lead to uncontrolled proliferation. DNA

damage response systems play a key role in preventing cancer growth, for example, the Mre11-Rad50-Nbs1 complex detects DNA double-strand breaks and activates ATM kinase, which phosphorylates CHK2, thereby stopping the G1 checkpoint to prevent cell division [18, 19].

The effects of Perifosine on the cell cycle were evaluated by analyzing the expression of genes coding for CCNA1, CCNA2, and CCND1, which regulate different stages of the cell cycle. CCNA1 plays a key role at the G1 and G2 checkpoints, where it interacts with CDK2 and CDC2 kinases to facilitate the cell's progression through these phases. HepG2 cells treated with IC25, IC50, and IC75 concentrations of perifosine showed altered expression of CCNA1, CCNA2, and CCND1. Up regulation of CCNA1 suggests a halt in the G1 or G2 phase of the cell cycle. The downregulation of CCNA2 at all concentrations further supports these cell cycle arrests. This inhibition aligns with the role of cyclin A2 (CCNA2) in the regulation of mitotic progression and its potential involvement in cellular stress responses [20]. The concentration-dependent inhibition observed in this study suggests that the compound may affect mitotic progression or induce cell cycle arrest, which could contribute to their anti-cancer effects.

The expression of CCND1 was also influenced by perifosine, with a noticeable increase at higher concentrations. The CCND1 gene, encoding cyclin D1, plays a pivotal role in regulating the transition from G1 to S phase of the cell cycle. The observed minimal changes at lower concentrations followed by significant induction at higher concentrations suggest a potential saturation point or maximum response level for these compounds. This pattern is consistent with the reported role of cyclin D1 in tumorigenesis and its modulation in response to cytotoxic agents [21]. Additionally, the 2.5-fold increase in CCND1 expression in IC50 and IC75-treated cells indicates a halt specifically in the G1 phase, as CCND1 interacts with the Rb protein to regulate this checkpoint.

The study demonstrated the anticancer potential of synthetic ALP perifosine against HepG2 liver cancer cells. The compound caused significant toxicity and altered expression of key cell cycle genes, suggesting its potential as antineoplastic and cytostatic agents. These findings warrant further investigation to explore perifosine effects in HCC cells, while including additional *in vitro* and *in vivo* studies to support future clinical evaluation. Provided with further evidence, perifosine may be a new addition to treatment modalities against liver cancer.

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RESEARCH ARTICLE

Effects of a ribosome inactivating plant protein (riproximin) on transcriptomic profile of apoptosis pathway genes in breast cancer cells

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Abstract

Background: Treatment options for breast cancer are limited, and the available possibilities are largely palliative in nature. In this context, searching for more effective therapeutic compounds is inevitable. Riproximin, a plant derived ribosomal inactivating protein, is one such kind of anticancer compound and has shown substantial anticancer effects while altering the major components of different molecular pathways including cell cycle, autophagy, cell stress and apoptosis. The purpose of this study was to investigate the effects of riproximin exposure on expressional levels of genes associated with apoptotic pathway in breast cancer cell lines.

Methods: In first phase, breast cancer cell lines (MDA-MB-231 and MCF-7) were exposed to riproximin, and toxic effects were identified by MTT dye reduction assay. Afterwards, the cell lines were exposed to riproximin in a separate experiment and expressional modulations of multiple genes were evaluated via real-time PCRs. Fold changes in this study were calculated by Livak method ($2^{\Delta\Delta Ct}$) while using untreated cells as controls.

Results: Proliferation (MTT) assay showed that riproximin induced promising cytotoxic effects in breast cancer cell lines. MCF-7 cells were more responsive towards riproximin exposure as compared to MDA-MB-231 cells. Expressional assessment of the selected genes confirmed the potential of riproximin to alter the levels in breast cancer cells. Specifically, in response to riproximin exposure, three most effectively induced genes in MDA-MB-231 cells were BIK (83fold), TNFSF9 (59fold) and NFKB1 (20fold). In MCF-7 cells, three most up-regulated genes after riproximin treatment were TNF (129fold), LTA (58fold) and TNFSF9 (17fold).

Conclusion: Riproximin bears significant cytotoxic potential against breast cancer cell lines. Expressional modulations in multiple apoptotic related genes are imposed by riproximin in the breast cancer cells. Further in *vitro* and in *vivo* studies are required to support the evaluation of riproximin in clinical settings against breast cancer cells, while targeting the apoptotic routes for cancer treatments.

Key Words: Breast cancer, Apoptosis, Riproximin, Protein, Ribosome inactivating

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INTRODUCTION

Breast cancer is the leading cause of cancer related mortalities worldwide. The most common type of breast cancer is ductal carcinoma. Other breast cancer types include ductal carcinoma in situ, invasive breast cancer and inflammatory breast cancer [1]. Histological type, tumor grade, and tumor stage are key parameters in diagnosis of breast cancer by using criteria as outlined in Nottingham Grading System. Treatment strategy is administered according to the subtype and stage of disease. There are different treatment strategies being used for breast cancer treatment which include chemotherapy, radiation therapy, hormonal therapy and surgery. Treatment options are limited for advanced stages of this cancer and the available opportunities are largely palliative in nature. In this context, searching for more effective therapeutic compounds is inevitable [2, 3].

Plants have been an attractive source of natural therapeutic compounds against various diseases including cancers. Plant-derived compounds can differentiate between cell types, being non-toxic to normal cells [4]. Almost a decade ago, a ~60 kDa active protein component was extracted and purified from the kernels of *Ximenia americana*, and was named as riproximin, a type two ribosome inactivating protein [5]. Ribosomes inactivating proteins (RIPs) are the plant proteins that stop protein synthesis by enzymatically damaging the ribosomes [6]. RIPs are widely distributed in nature and originate from plants. In addition, other resources include microorganisms, algae, mushrooms and even insects. Based upon physical properties, RIPs are classified into three main

groups i.e., type 1, 2 and 3. Different types of RIPs have been studied, and these proteins negatively affect the growth of tumor cells *in vivo* and *in vitro* [7-9].

Riproximin belongs to type 2 RIP with two polypeptide chains A and B. A-chain has N-glycosidase activity while B-chain has lectin domain for binding purposes. The mechanism of working of riproximin is through depurination of 28s RNA of eukaryotic ribosome. This depurination of 28s rRNA results in transcriptional arrest. Additional mechanisms for riproximin mediated signaling pathways that lead to apoptosis include ribotoxic response, stress induced mitochondrial pathway, down regulation of anti-apoptotic factors, NAD+ depletion of PARP hyper-activation and DNA damage due to nuclease activity. Riproximin is used to trigger apoptosis as cancerous cells evade apoptosis via various mechanisms. Triggering apoptosis is an alternative to chemotherapy which has toxic effects on both cancerous and normal cells while riproximin is non-toxic to healthy cells [10].

Anticancer effects of this protein have been evaluated against a variety of cancer cell lines including primary and metastatic cancer cells. Over the years, a series of experiments showed significant antineoplastic potential of riproximin in *in-vitro* studies. Recently, the antineoplastic effects of riproximin in breast and colorectal cancer cell lines have been evaluated by various functional assays. The results highlighted the significant inhibition of proliferation, migration, colony formation, induction of apoptosis and S-phase cell cycle arrest in selected cell lines. At molecular levels, riproximin modulates multiple signaling cascades leading to cytostatic and apoptotic effects in human breast and colorectal cancer cells [11, 12]. Avoiding the apoptotic routes is a hallmark of cancer cells and targeting such sensitive aspects via natural compounds could be a highly effective anticancer approach. Keeping in mind the apoptosis inducing ability, the current study was conducted to reveal the effects of riproximin on expressional modifications of apoptotic related multiple genes in breast cancer cells. Furthermore, the expressional modulations were used to design signaling cascades being affected by riproximin in the target breast cancer cells to induce death mechanisms.

METHODS

Cell culture

Two Human breast cancer cell lines (MDA-MB-231 and MCF-7) were maintained in cell culture medium RPMI-1640, supplemented with 10% of FBS, 2mM L-glutamine, 100µg/ml of streptomycin, 100IU/ml of penicillin with standard humidification conditions of 5% CO2 and temperature of 37^0C. Routine culturing of cells was done (cells passaging 2-3 times/week) to maintain logarithmically growing cell population.

MTT Dye Reduction Assay

Cultured cells were treated with serial dilution of riproximin at pre-optimized cell densities (4000cells/well) in 96-well plates and exposed to various concentrations of riproximin (0.05-50ng/ml) for three-time intervals i.e., 24, 48 and 72 hours and toxic effects of riproximin against breast cancer cells were studied by a calorimetric MTT dye reduction assay. After the treatment intervals, MTT solution (10mg/ml in PBS) was added to the wells followed by incubation at standard conditions for 3 hours. Formazan crystals, formed by the viable cells, were dissolved by addition of 50µl of DMSO in each well. Optical densities of the samples were measured by using an ELISA reader at 540 nm and 645 nm reference filter. Cell survival rates were calculated as percentage of the untreated cells while inhibitory concentrations (ICs) were calculated by GraphPad Prism 9 software.

Treatment with Riproximin

For expressional profiling, cells were cultured in 6-well plates at a density of 150,000 cells/well/ 2ml of medium and next day, treated with three different concentrations of riproximin (1-50ng/ml) for 48 hours. Afterwards, the cell palettes were collected by trypsinizing the cells with 0.05% solution of trypsin-EDTA and collected by centrifugation at 1500-2000rpm for a duration of 5min followed by storage at -80^0C. Likewise, the cell palettes of untreated cells as controls were also collected and stored in similar conditions. These two cell lines were comprised of eight samples in total i.e., two controls (untreated MDA-MB-231 and MCF-7 cells) and six riproximin treated samples of MDA-MB-231 (5, 10, and 50ng/ml) and MCF-7 cells (1, 5, and 20ng/ml)

RNA Extraction and Quantification

Following the exposure period of cultured cells with riproximin total RNA was extracted (Thermo Fisher Scientific, Cat#K0731) from control and treated cell lines and was quantified by placing 2µl of extracted RNA on Nanodrop ND2000 designated area and readings were taken as prescribed protocol.

cDNA Synthesis & Verification

A total of 1500ng of extracted RNA, was used to synthesize the cDNA (40µl) by using Reverse Transcriptase, OligodT and dNTPs following the protocol of selected kit (Thermo Fisher Scientific, Cat#K1622). For verification purposes, a PCR based amplification of a reference gene (GAPDH) was done of prepared cDNA samples and amplified products were loaded on 2.5% agarose gel and visualized by gel electrophoresis.

Primer designing and Optimization

A total of ten genes related to apoptosis pathway were selected and primers were designed by choosing gene specific sequences from NCBI Gene bank and using Primer 3 plus (Table 1) and optimized via gradient PCR methodology. After some consistent efforts, primers were optimized for their respective appropriate master mix requirements and cyclic conditions.

Real Time PCR and Data Analysis

By using cDNA generated from cell lines (MDA-MB-231 and MCF-7) untreated and treated with different concentrations of riproximin, qRT-PCR was performed by using SybrGreen fluorescence dye for selected optimized 10 genes. After normalization of data sets by using reference gene, the data was analyzed by calculating fold changes using Livak 2-ΔΔCT method by comparing Cq (quantification cycle) values of experimental (riproximin treated) and untreated control samples.

Table 1: Primer sequence for amplification of selected genes

Gene	Primer Sequence (F)	Primer Sequence (R)
BAK	AGAGTTCCAGACCATGTTGC	CATGCTGGTAGACGTGTAGG
BCL10	ACTGAAGTGAAGAAGGACGCC	CAACAAGGGTGTCCAGACCT
BIK	GTCCTGGGTGTCCTGCGAA	AAGATAACAGCAGCAGGCCG
FASLG	CTGGGGATGTTTCAGCTCTTC	CTTCACTCCAGAAAGCAGGAC
GAD45A	AACGGTGATGGCATCTGAAT	CCCTTGGCATCAGTTTCTGT
LTA	CTCACCTCATTGGAGACCCC	CCACCTGGGAGTAGACGAAG
NFKB1	CCTACGATGGAACCACACCC	ATCTGCTCCTGCTGCTTTGA
TNF	CCTGCTGCACTTTGGAGTGA	GAGGGTTTGCTACAACATGGG
TNFSF9	CTTCCTCACGCTCCGTTTCT	ACTGGTCTCATAAATGGTTGTTTGA
TRAF3	GTCCTGACAGAAGAGAACTCC	TTTAGCGGCGGGTTAGTCTG

RESULTS

Toxicity of Riproximin

Toxic effects of riproximin against breast cancer cell lines MDA-MB-231 and MCF-7 were studied by MTT dye reduction assay and ICs were identified with the help of GraphPad Prism 9 software. Among the two cell lines, metastatic MDA-MB-231 cells were less responsive as shown by higher IC50 especially after 48 hours of exposure time. However, the differences between the sensitivity of the two cell lines were reduced after longer exposure (72 hours) with riproximin. IC50 values for selected breast cancer cell lines MDA-MB-231 and MCF-7 are given in Table 2.

RNA Extraction and cDNA Synthesis

RNA was extracted from untreated control and treated breast cancer cell lines (MDA-MB-231 and MCF-7) and measured by nanodrop technology. Extraction procedure was successful as a good quantity (55-357ng/µl/sample) and quality (260/280 ratio: 1.9-2.0) of RNA was extracted from the samples. Following the protocol of selected reverse transcriptase enzyme kit (Thermo Fisher Scientific, Cat# K1622), cDNA was synthesized by using extracted total RNA and verified by PCR based amplification of a reference gene (results not shown here).

Expressional Analysis of Genes

Effect of riproximin on the selected genes (BIK, BAK1, BCL10, FASLG, GAD45A, LTA, NFKB1, TNF, TNFSF9 and TRAF3) in breast cancer cell lines (MDA-MB-231, and MCF-7) were investigated after exposure with three different concentrations of riproximin. The modifications in four (LTA, TNF, TNFSF9, TRAF3) were concentration dependent on both cell lines, which reflected continuous induction in response to higher concentrations of riproximin exposure. NFKB1 and BIK show almost the same trend as highest alterations were reflected in response to the highest riproximin concentration applied.

In addition to this, there were some cell specific responses as well as shown by concentration dependent induction of BAK1 was observed for MDA-MB-231 cells, while MCF-7 cells did not follow this pattern. In contrast, BCL10 and FAS genes were highly induced against the lowest concentrations of riproximin (1ng/ml) in MCF-7 cells, while the effects were minimized at highest concentrations (20ng/ml). Overall modifications in the genes are shown in Figures 1.

Table 2: IC50 concentrations (ng/ml) after 48 and 72 hours of treatment.

	Breast Cancer Cell Lines	
	MDA-MB-231	**MCF-7**
48 h	10.2ng/ml	1.8ng/ml
72 h	2.9ng/ml	1.2ng/ml

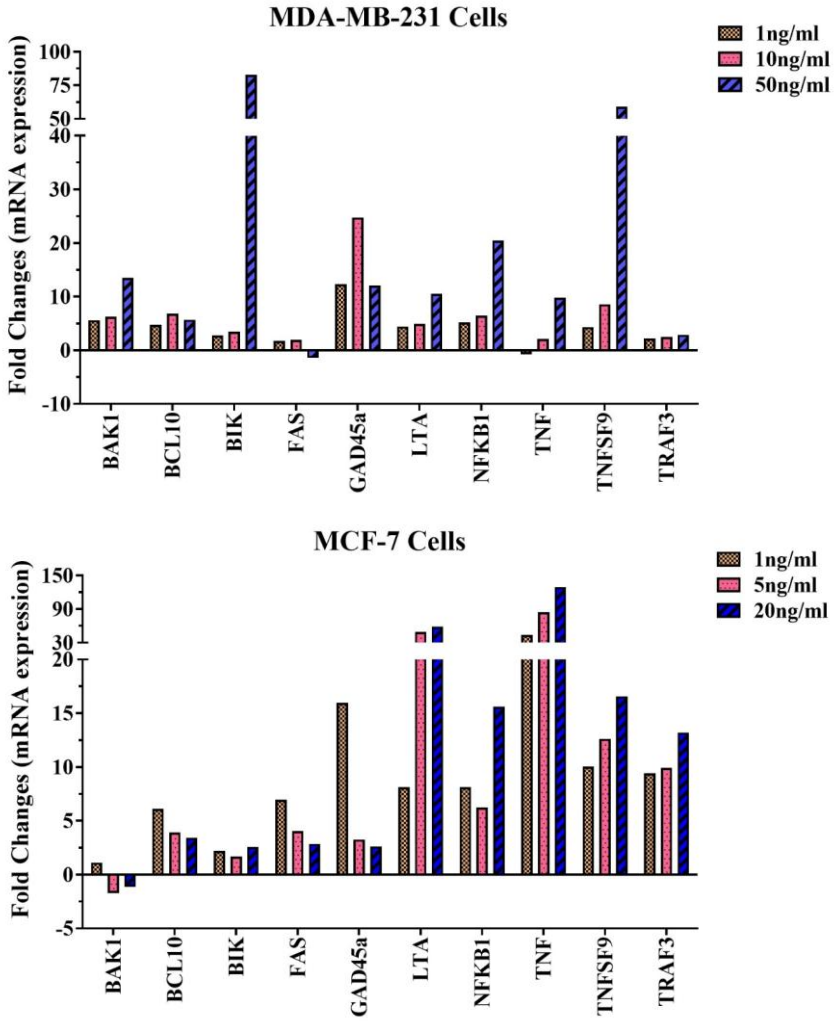

Figure 1: Fold changes of selected genes in MDA-MB-231 and MCF-7 cells. The cells were exposed to riproximin, and expressional changes were determined by using real-time PCRs and Livak method.

DISCUSSION

Cancer is a dreadful disease, causing a great deal of mortality and morbidity throughout the world. There are more than 100 types of cancers known and breast cancer is the second leading malignancy among them. Management of breast cancer is challenging as treatment strategies are not sufficient to control the burden of breast cancer and available treatments are costly and impose huge side effects as well. For this reason, focus should be shifted towards novel naturally occurring therapeutic agents to control the side effects and plants are being used for centuries against various diseases including cancers. Among these seeds of *X. americana* have been used by the local inhalers for treatment of cancers in African countries [12]. Active fractions of these seeds named riproximin have been tested against various cancer cell lines and animal models for their anti-neoplastic effects [11-14]. The purpose of this study was to evaluate the effects of riproximin on apoptosis related genes in breast cancer primary (MCF-7) and metastatic (MDA-MB-231) cell lines.

Initially, MTT dye reduction assay was used to determine and confirm the sensitivity of breast cancer cell lines towards riproximin by exposing the cell lines to increasing concentrations of riproximin (up to 50ng/ml) for 24-72 hours. According to the results, primary cell lines MCF-7 were more responsive as compared to metastatic MDA-MB-231 cell lines towards riproximin which is in line with already available literature [11]. This is indicated by IC50 values at 48 and 72 hours of exposure periods which are 1.8 and 1.2ng/ml for MCF-7 and of 10.2 and 2.9ng/ml for MDA-MB-231. This difference in sensitivity may be due to certain factors like affinity difference of riproximin towards both cell lines or the molecular difference between the two breast cancer cell lines (ER/PR +ve/-ve or presence of wild or mutated type P53).

To determine the effect of riproximin on apoptosis related genes in two breast cancer cell lines, the cells were exposed to different concentrations of riproximin (MDA-MB-231: 1, 10 and 50ng/ml, MCF-7: 1, 5 and 20ng/ml) for 48 hours. After the exposure period, RNA was extracted from treated cell lines, quantified and converted to cDNA for subsequent real time amplification by using gene specific primers. Results of these expression profiling experiments showed comparable outcomes which in turn demonstrate that riproximin has effective potential to induce apoptosis related genes in primary and metastatic breast cancer cells. However, the important point is that the level and type of apoptosis related genes induced by riproximin in these cells varied substantially. For example, BIK and GADD45A were one the most effectively induced genes in MDA-MB-231 cells. Among these, BIK is a BCL-2 interacting killer pro-apoptotic protein and plays an important role in initiating apoptosis [15]. This, in turn shows that riproximin has potential to interact with intrinsic arm of apoptosis and can induce vital mitochondrial associated gene families. GADD45A is a major indicator of cell stress and leads the way to induce apoptosis by fragmentation of DNA and nuclei [16]. Current data shows that riproximin can act like a cell stress inducer and can up-regulate the related gene families like GADD. In MCF-7 cells, LTA and TNF were the most effectively induced genes in response to riproximin exposure. LTA and TNF have substantial importance in survival and death mechanisms [17]. Induction of these genes shows that riproximin has potential to alter the expression of master regulators to induce apoptotic process in breast cancer cells. In addition to above mentioned genes, NFKB1, a very important transcription factor was shown to be upregulated effectively in both breast cancer cell lines. This transcription factor has tremendous importance for regulating the release of cytokines, immune responses and stress mechanisms. Further studies should also be aimed at investigating the induction of NFKB1 via riproximin in breast cancer cells as it could be a vital arm for inducing anticancer effects clinically. In a nutshell, riproximin bears significant cytotoxic potential against breast cancer cell lines. Expressional modulations in multiple apoptotic related genes are imposed by riproximin in the breast cancer cells. All in all, data reflected that riproximin is a promising antineoplastic compound with substantial potential to alter genetic expression and needs due attention for its considerations as anticancer agent.

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RESEARCH ARTICLE

Neureglin 3 gene intronic polymorphisms rs10883866, rs6584400, rs1937970 and rs677221 in patients with Schizophrenia

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Abstract
Background: Schizophrenia is a progressive disease presenting with insufficiencies in thought processes, perceptions, and emotional responsiveness with early onset. There are no laboratory tests or specific biomarkers established so far to diagnose the disease, so clinical and psychiatric analysis are used worldwide. Abnormal neurodevelopment, genetic predisposition and environmental factors are the main factors associated with this disease. The Neuregulin 3 *(NRG3)* gene encodes the Neuregulin 3 protein which binds to the extracellular domain of ERBB4 receptor tyrosine kinase and controls the proliferation, differentiation, and migration of neural progenitor cells.
Methods: Purpose of this study was to find the *NRG3* gene polymorphisms i.e., rs10883866, rs6584400, rs1937970 and rs677221 in Schizophrenic patients (n=114) and then compared with healthy controls (n=114). For this purpose, blood samples were taken after the informed consent of patients and the controls followed by DNA extraction and genotyping through PCR-RFLP and PCR-ARMS.
Results: The minor allele frequencies of the SNPs rs10883866, rs6584400, rs1937970 and rs677221 in schizophrenic patients were 21.1%, 28.1%, 68.1% and 62.2% respectively. The SNPs rs10883866 and rs6584400 in protective effect toward the Schizophrenia whereas, the other two SNPs showed any statistically significant difference in the allelic distribution between cases and controls. Haplotype block analysis revealed that C-G haplotype in block 1, A-G in block 2 had frequencies of 62.4% and 60.7% in cases. The haplotype G-A in block 1 showed the protective effect towards the disease Schizophrenia with the *p-value* 0.011.
Conclusion: *NRG3* has association with the disease schizophrenia. More studies should be conducted including different phenotypic traits of schizophrenia and meticulous clinical evaluation of the patients with larger sample size is suggested to find out the association of NRG3 gene with the schizophrenia.
Key Words: Schizophrenia, Neuregulin 3 gene, Polymorphism

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INTRODUCTION

Schizophrenia disease is presented with insufficiencies in thought processes, perceptions, and emotional responsiveness. The disease has an onset usually in late adolescence or early adulthood [1]. Schizophrenia is diagnosed clinically by psychiatric analysis and by observing patient's abnormal social behavior. There are no laboratory tests or specific biomarkers that can be used to make a diagnosis of this disease [2]. Abnormal neurodevelopment, genetic predisposition and environmental factors are considered to be the main factors associated with this disease [3]. The developmental process suggests that environmental insults, early during the development of fetus, such as viral or bacterial infections, nutritional deficiencies, exposure to neurotoxins and obstetrics complications may result in the person developing neuropsychiatric disorders including schizophrenia later in life [4].
The neuro-developmental theory of schizophrenia suggests that the basic pathology of schizophrenia occurs during the development of brain and that these developmental brain abnormalities do not become evident until a person reaches adolescence [5]. This is supported by the detection of neuroanatomical and cyto-architectural abnormalities in the brains of patients. Problems of neuronal migration are seen in the brains of these patients as is evident by out-of-place and grouped neurons that are particularly seen in the entorhinal cortex, suggesting an early developmental abnormality. Smaller cell bodies and fewer dendritic spines are seen in pyramidal neurons of the hippocampus and neocortex of schizophrenia patients [6, 7].
Heritability of schizophrenia is strongly exhibited by family, twin and adoption studies. Inheritance mode is complex and does not follow the Mendelian law. Family studies demonstrated a higher risk of developing schizophrenia in the individuals who have first or second degree relative affected with this disease as compared to general population [8]. It has been reported that the risk of developing schizophrenia in

second degree relatives is about 2–4 %, which increases in first degree relatives to 10–15 % and up to 45% in monozygotic twin [9]. In recent years, due to advances in DNA analysis technology, there has been a rapid advancement in understanding of the genetic basis of schizophrenia. Several diseases are associated with DNA variations have been identified by international consortia working on genetics of this disease. These variations are spread all over the human genome and are present in genes that regulate specific function. Although each individual variant itself has a little effect, it has been predicted that common variations when taken as a whole account for about half of the genetic variance in schizophrenia.

The neuregulin proteins are expressed in neurons and perform a wide variety of functions in the developing nervous system. Neuregulin 3 (*NRG3*) belongs to this family and is present on the long arm of chromosome 10 (10q23.1) [10, 11]. *NRG3* gene has 12 exons: first five of these exons encode the extracellular epidermal growth factor domain of the protein, whereas exons 8 to 12 encode the cytoplasmic portion of the protein. In both fetal and adult human brain, thirteen different *NRG3* splice variants have been identified that classify this protein into 4 different classes. Differential regulation of *NRG3* isoforms is considered to affect neurodevelopmental pathways and play a role in the development of schizophrenia [12]. It has been found that expression of Class 1 isoforms of *NRG3* is increased to 40% in the dorsolateral prefrontal cortex of schizophrenia patients as compared to controls. Similarly, the expression of Class IV *NRG3* isoforms is also increased by 50% in individuals with schizophrenia as compared to normal controls, while Class II and III transcripts did not show differences in expression. Neuregulin are reported to be important in regulating the growth and differentiation of glial, epithelial, and muscle cells [13, 14].

NRG3 is a growth factor that binds to the extracellular domain of ERBB4 receptor tyrosine kinase. This binding activates tyrosine phosphorylation of ERBB4 receptor and modulates the neuronal migration and differentiation [15]. *NRG3* plays pleiotropic roles in the development, plasticity of the brain and is essential for the embryonic cerebral cortex development. It does so by controlling the proliferation, differentiation, and migration of neural progenitor cells [16]. The four SNPs analyzed in the present study were rs10883866, rs6584400, rs1937970 and rs677221. The two SNPs rs10883866 and rs6584400 are located in intron 1 while rs1937970 and rs677221 lie in intron 2.

METHODS

Clinical Sampling

A total of 114 diagnosed schizophrenic patients fulfilling the criteria of DSM IV and of both genders were recruited from Department of Psychiatry in the tertiary care hospital of Lahore, Pakistan. For comparison purposes, 114 healthy subjects with no personal and family history of schizophrenia or any other psychiatric disorder were also included in the study as healthy control subjects. After an informed written consent, blood samples were collected from the schizophrenic patients and normal subjects in EDTA containing vials.

Genotyping of Samples

The DNA was isolated by salting out procedure and estimation of its quality and quantity was done using Nanodrop. Genotyping for SNPs rs10883866 and rs658400 was done by PCR-RFLP using the TspR1 and Hinf1 restriction enzyme method as mentioned in supplementary Table 2. The Components and concentrations of ARMS-PCR reaction are given in supplementary Table 3. The SNPs rs1937970 and rs677221 were genotyped by the ARMS-PCR. The primer sequences are given in supplementary tables 4-5. The components and concentrations of PCR and RFLP reaction are given in supplementary Table 2 and 1. PCR-RFLP and ARMS-PCR products were resolved at 2% while ARMS-PCR products were resolved on 3% TAE agarose gel along with 50bp DNA size marker based on molecular weight.

Data Analysis

Hardy–Weinberg equilibrium was tested to check the deviation of genotype count using a chi-square goodness-of-fit test using OEGE-Online Encyclopedia for Genetic Epidemiology Study. Chi-square test evaluated statistical differences in genotypic and allelic distribution between schizophrenia patients and control subjects at significance level of 0.05. The effect of different alleles was evaluated by calculating odds ratio (OR) and their 95% confidence intervals (95% CI) using IBM SPSS software (version 20.0, SPSS Inc). *p*-value < 0.05 was considered statistically significant. Genetic modeling was done using SNPStats software and Haplotype analysis was done using SHEsis online software.

RESULTS

Demographics of Cohort

There were 57.7% male patients and 42.3% were females. The age of the patients ranged from 18 years to 80 years with a mean age of 35 ±11.6 years. The majority of patients (62.0%) were in the age group of 25-40 years and in this age group, percentage of female patients (70.0%) was greater than males. Majority of patients (70.8%) were product of consanguineous marriages. According to their age groups, they were further stratified into three groups, below 25 years, 25-40 years, and above 40 years. The mean age of the study population was 35±11.6 years. Age distribution showed that 62.0% of patient's ages were 25-40 years. However, when male to female ratio was compared to each age group, it was found that around 70.0% females (n= 35) were affected during the age group 25-40 years.

Genotyping for *NRG3* SNPs by PCR-RFLP

Supplementary figures 1-4 show gel images of the genotyping results for SNPS. The allelic and genotypic frequencies of the four SNPs are given in the Table 6 and 7. The heterozygous CG genotype of rs10883866, rare homozygous AA genotype of rs6584400 and rare CC homozygous genotype of rs677221 showed the protective influence of genotype over schizophrenia with ODDs of, because its frequency in control group was significantly more than patient population with ODDs ratios and CI as (0.32 [0.20-0.52] *p=0.042,* (0.390 [0.201-0.756] *p=0.014 and* 0.258 [.105-.633] *p-value 0.0013* respectively.

Haplotype Analysis and Pairwise Linkage Disequilibrium:

Linkage analysis was done for all the possible pairwise combinations of 4 SNPs with minor allele frequency≥ 1%. Figure 1 exhibits the visual depiction of the degree of LD which was determined by the standard disequilibrium coefficient D' and r^2 values. Two LD blocks were defined using these D' and r^2 values. Each block contained two SNPs i.e. rs10883866 and rs6584400 were included in block 1 whereas rs1937970 and rs677221 were present in block 2. The LD plot showed that the SNPs within each block were moderately linked as shown in Figure 1(A) and (B).

Haplotype block analysis revealed eight major haplotypes, four for the two SNPs of each block as shown in the supplementary Table 8. In block 1, C-G haplotype was the most occurring with the frequency of 62.4% in cases and 46.8% in controls whereas, in block 2 A-G was the most frequent haplotype of all with the frequency of 60.7% in cases and 48.52% in controls. The haplotype G-A in block 1 showed the protective effect towards the disease schizophrenia with the *p-value* 0.011. In block 2, no single haplotype showed any effect toward the disease's phenotype. The major haplotypes of each block, their corresponding frequencies and the odds ratios are described in supplementary Table 8.

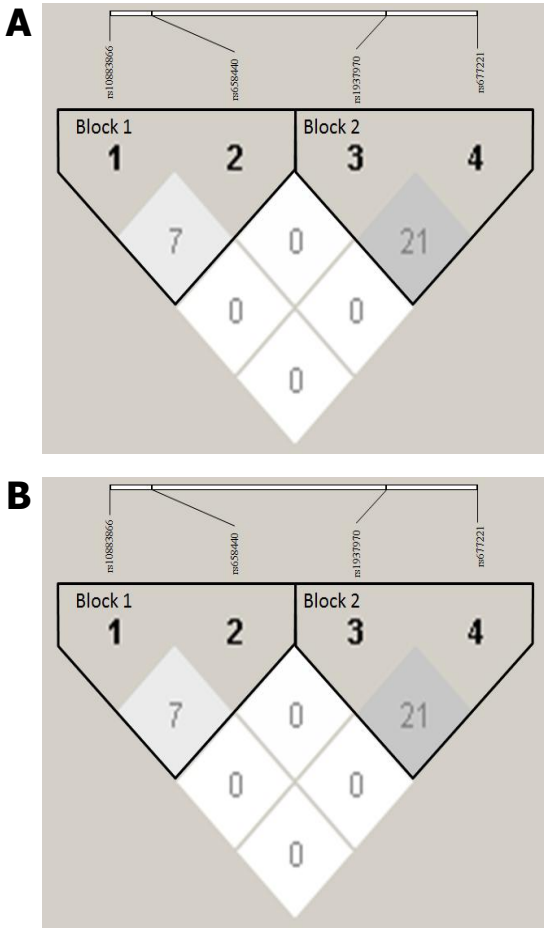

Figure 1: Linkage disequilibrium analysis for NRG3variants rs10883866 (1), rs6584400 (2), rs1937970 (3) and rs677221 (4). White bar indicates the span of NRG3 gene area having these four SNPs. The two pentagons represent the two blocks and the boldfaced numbers in blocks represent the htSNPs. Figure 3.7 (A) presents the LD plot constructed based on D' values and the level of LD is indicated with the color scheme given at its bottom right. Figure 3.7 (B) shows the LD plot based on r^2 values

DISCUSSION

Linkage studies in different populations have identified the 10q22-q23 chromosome region as potentially harboring risk genes for schizophrenia [17]. This region includes members of the neuregulin family, which comprises four epidermal growth factor-like molecules: *NRG1, NRG2, NRG3, and NRG4* [18].These molecules interact with the ERbB receptor tyrosine kinase family, and among them, *NRG1* and *NRG3* have been most strongly implicated in schizophrenia pathogenesis [19]. *NRG3*, a key member of the neuregulin family, binds specifically to the ErbB4 receptor and activate through ligand-stimulated phosphorylation [20]. Given with its predominant expression in brain regions such as the hippocampus, amygdala, and thalamus, *NRG3* plays a crucial role in neuronal development, particularly in cortical cell migration and patterning during embryonic brain formation [21].

This variant has been the focus of increasing attention in the field of schizophrenia research, as it has been consistently associated with the disorder across multiple populations and ethnic groups [22]. The rs10883866 variant is thought to play a role in the regulation of gene expression, potentially impacting the function of genes involved in neurodevelopment, neurotransmission, and other cellular processes relevant to the pathophysiology of schizophrenia [23, 24]. Beyond genetic contributions, emerging evidence suggests that epigenetic mechanisms, such as DNA methylation, may also contribute to the association between rs10883866 and schizophrenia [24].

The non-association of rs10883866 with schizophrenia in the present study aligns with findings from the Han Chinese and Ashkenazi Jewish populations. However, both studies demonstrated that when analyzing "delusion" as a quantitative trait of schizophrenia, rs10883866 showed significant association with this clinical feature. The present study focused on schizophrenia as a discrete phenotype and did not investigate specific clinical traits, which could explain the lack of association. In contrast, research on White Americans of European ancestry revealed an association between rs10883866 and both schizophrenia risk and the severity of delusions, along with increased expression of *NRG3* isoforms in the brains of affected patients [25]. Similarly, Australian studies found significant associations between rs10883866 and schizophrenia subtypes characterized by delusions and relatively intact cognition [26].

Regarding rs6584400, the current study found no association with schizophrenia, corroborating findings in Korean population [27]. However, studies in Han Chinese, Ashkenazi Jewish, and European populations reported associations between rs6584400 and delusional traits in schizophrenia [10, 14]. Notably, in German patients, rs6584400 was significantly associated with psychotic symptoms and cognitive performance [28]. These findings suggest that while rs6584400 may not contribute directly to schizophrenia, it could influence specific cognitive or behavioral traits. In the case of rs677221 and rs1937970, the present study found no significant association with schizophrenia, consistent with previous research on White American populations [25]. However, studies in Han Chinese populations reported that rs677221 remained associated with schizophrenia after multiple testing corrections [14], highlighting the variability in genetic associations across populations.

Overall, the current study adds to the evidence suggesting that *NRG3* SNPs may not be directly associated with schizophrenia in populations. Genetic heterogeneity, population-specific factors, and methodological differences likely contribute to the discrepancies observed between studies. Additionally, it appears that *NRG3* SNPs may be more strongly linked to specific schizophrenia traits, such as delusions, rather than to the disorder itself. Future research with larger sample sizes and comprehensive clinical assessments of schizophrenia traits is needed to clarify the role of *NRG3* in schizophrenia and identify other potential genetic contributors to this multifactorial disorder. Provided with further evidence, genetic testing covering NRG genes may provide an instrumental way forward in diagnosis and pathogenesis assessment of schizophrenia.

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Competing Interests: The authors declare that they have no conflict of interest.
Data Availability Statement: Datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Note: Supplementary data is available online.

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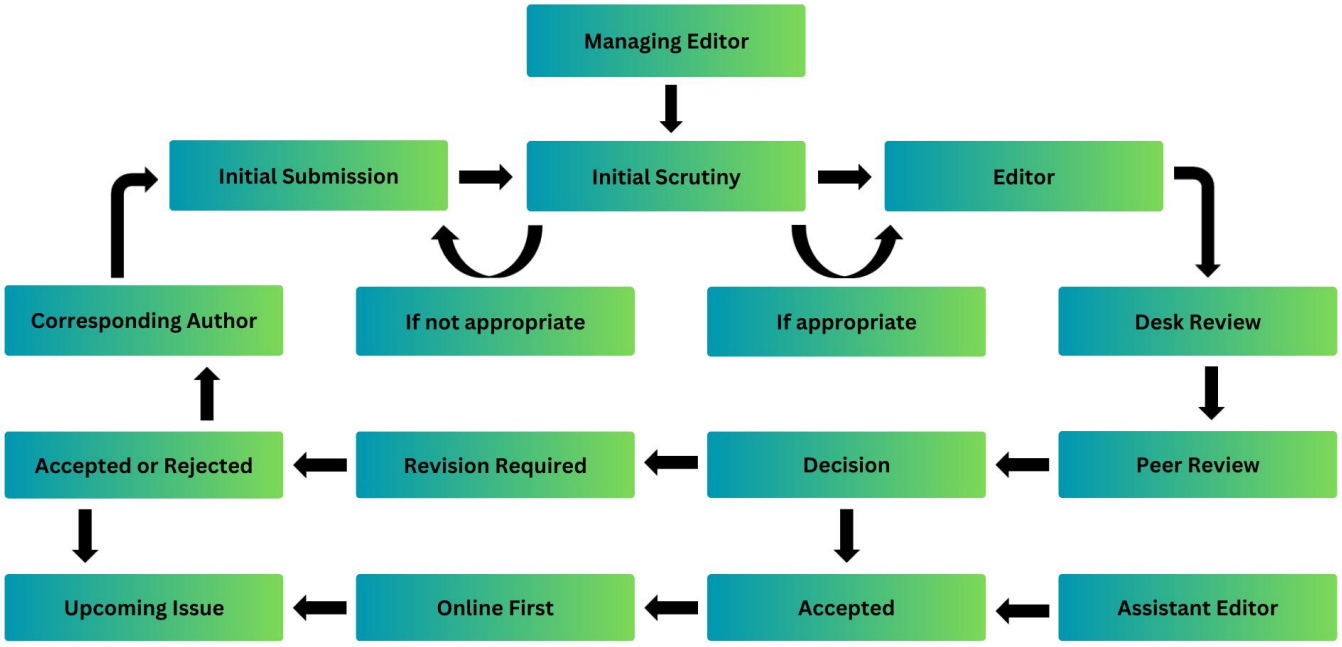

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