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EXPERIMENTAL STUDY ON CRISPR-Cas9 KNOCKOUT SCREENS AT THE GENOME SCALE FOR NASOPHARYNGEAL CARCINOMA

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ABSTRACT

A genome-scale CRISPR-Cas9 knockout screening may present novel perspectives on the currently undetermined pathway in nasopharyngeal cancer (NPC). To establish a basis for additional investigation into the functional mechanism of NPC, our goal was to perform a screening of its functional genes. Second-generation sequencing technology was employed with CRISPR-Cas9 library lentivirus screening in NPC cells to uncover functional genes, subsequently confirmed in NPC cells and patient tissues. In the King Edward Medical University, Mayo Hospital, Lahore laboratory, eleven radiosensitive and radioresistant genes were examined in 2023. Among these genes, in the radioresistant NPC cells, the expression of TOMM20, CDKN2AIP, SNX22, and SP1 was higher and that of FBLN5, FAM3C, MUS81, and DNAJC17 was considerably reduced ($p < 0.05$). In C666-1R, CALD1 was substantially upregulated. Additionally, we discovered that the deletion of genes facilitated the growth of NPC cells, whereas SP1 exhibited the opposite effects ($p < 0.05$). Using tissues from NPC patients, this outcome was confirmed. Furthermore, KEGG analysis suggested that the TGF-β signalling pathway and the Fanconi anaemia pathway may have played a role in NPC. There are nine genes under investigation. To increase the effectiveness of treatment for NPC, genome-scale CRISPR-Cas9 knockout screening for genes in NPC may offer fresh perspectives on the mechanisms.

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1. INTRODUCTION

A malignant tumour called nasopharyngeal carcinoma (NPC) develops on the upper and lateral border of the nasopharyngeal cavity. The tumour originates from the epithelial cells that cover the nasopharyngeal mucosa. Non-small cell lung cancer (NPC) has a unique global distribution and a complex cause. Pakistan, Western Canada, Northern and Northeastern Africa, China, and Southeast Asian nations seem to have higher occurrence rates. The World Association for Investigation on Oncology reports 80,008 new NPC fatalities and 133,354 additional cases in 2020. Geographically restricted to East and Southeast Asia, it is likely to exhibit a yearly tendency towards growth [1- 3]. Radiation therapy, the primary management option for non-proliferative cancer (NPC) due to its high sensitivity to ionizing radiation (IR), causes cell death by triggering mortality signals through the production of reactive oxygen species (ROS), destruction of DNA, and stress responses. The inherited characteristics of the conditions, external factors, and exposure to EBV interact to cause the illness. External variables have been suggested to play a role; these include significant alcohol intake, cigarettes (2–6 times greater risk), and dietary components comprising nitrosamines. Furthermore, the inherited makeup of the affected populations is also crucial, as seen by the significant prevalence in the Chinese populace, which accounts for up to 18% of every case of cancer in some regions. There has been significant evidence linking genetic predisposition to EBV infection with the illness [4].

On the other hand, a tiny percentage of cancerous cells can survive disruption by triggering defence mechanisms like DNA repair and the unfolded protein response (UPR) inducing autophagy. For specific individuals, a persistent or persistent illness that results in unsuccessful therapy is still primarily caused by radioresistance [5]. Thus, it is critical to comprehend the fundamental causes of radioresistance and devise a strategy to enhance therapy efficacy. Due to its physical characteristics and elevated radiation sensitivity rad, radiation therapy is the primary treatment for NPC [6]. Considering a 60% 5-year life expectancy percentage, the outcome for locally progressed NPC remains unsatisfactory notwithstanding advancements in radiation therapy and technological upgrades [7], [8]. Bacteria employ CRISPR, an innate immunological mechanism, to prevent the introduction of foreign genomic information. Its benefits include accuracy, effectiveness, ease of use, and the capacity to generate editing outcomes at target locations [9]. The CRISPR-Cas9 gene-editing system consists of the single guide RNA (sgRNA) and the endo nuclide-functioning Cas9 protein (or additional proteins that operate similarly). Disabled caspase-dead Cas9 mutants (dCas9) merge, engage transcription molecules, and operate on transcription start sites (TSS) to regulate gene transcription. When sgRNA targets single genes, CRISPR is an effective gene-editing tool [10]. When sgRNA targets whole genome sequences, CRISPR's effectiveness as a genomic-wide screening technique is increased [11], [12]. 2012, the first article describing CRISPR-Cas9 gene editing in mammalian cells was published [13]. A new generation of gene-editing technology, CRISPR-Cas9, is effective and efficient. It has been effectively employed to alter genes [14], fruit flies [15], mice [16], and other plant models [17]. The CRISPR-Cas9 technology has advanced significantly in recent years. Higher specificity CRISPR cleavage targets can cover the entire genome by creating sgRNA hybridization libraries. Therefore, by causing gene mutations and activating transcription, CRISPR-Cas9 can be employed for functional acquisition and functional deletion screening [18]. NPC radiation resistance is a significant problem that hurts patient prognosis. Traditional research based on particular gene function analysis has identified numerous routes or mechanisms linked to the radiosensitivity of non-proliferative cancers (NPCs) [19- 22]. There isn't a systematic method for determining therapy for NPC at present. Through the meticulous and exhaustive examination of the gene functions and processes associated with radiosensitivity

and radioresistance, omics-based screening analysis technology can provide a more profound knowledge of the biological processes behind radiosensitization and radiation resistance. The Cas9 collection is an essential asset for advanced screening using the function of genes. However, the use of the CRISPR-Cas9 library to screen NPC for radiosensitivity and sexual resistance genes is not well documented in previously published studies. Implementing a genome-wide CRISPR-Cas9-sgRNA library virus, radiosensitive and radioresistant functional genes in NPC were tested in this study. It provides an arrangement for rigorously examining the processes of radiosensitivity and radioresistance of the NPC.

2. RESEARCH METHODOLOGY

At the Department of Oncology and Radiotherapy, King Edward Medical University, Mayo Hospital, Lahore, tissue samples were taken from 15 patients with non-small cell lung cancer (NPC) who were resistant to radiation and 15 patients with NPC who were susceptible to it. Patients classified as radiosensitive were individuals who were in complete remission (CR) or partial remission (PR) for three months after radiation, whereas patients classified as radioresistant were those who were evaluated as stable disease (SD) or progressive disease (PD) at three months after radiation therapy and recurrence within six months. Samples were gathered before the patients received radiation. Every tissue sample was encased in paraffin and preserved in 4% formalin. The study was ethically approved by the ethical committee of King Edward Medical University, Mayo Hospital, Pakistan, with 23KEMU202306. Each patient provided written informed authorization. Every procedure was carried out in compliance with the applicable rules and regulations. A genome-wide CRISPR-Cas9 gene knockdown screening was conducted on NPC cell lines C666-1. One thousand eight hundred sixty-four microRNA genes and 19,050 encoding genes were found in the collection. The cells were infected with the Cas9 library virus and split into three groups for radioresistance screening. High-throughput sequencing analysis was performed after PCR amplification of the sgRNA coding area using genomic DNA from live cells. The experiment was set up with two-time point samples, with early library infection stage samples kept for trend correction. Puromycin was applied to the cells at varying concentrations for 0–48 hours. Bioinformatics was used for the evaluation. The primary data from sequencing were taken out and modified with inadequate quality to obtain accurate information. After quality assurance, the appropriate sequencing expression frequency was obtained and compared to the sgRNA sequence of the library gene. Every sgRNA was then purified for analysis. The efficient genes showed a particular level of enhancement in the cell specimens acquired 7 days after irradiation. The study aimed to increase the radiation doses to C666-1 and 5-8F cells to produce radiation-resistant cells, namely C666-1R and 5-8FR. These cells underwent five rounds of testing and increased enormously. Cas9 technology produced stable cell lines with minimal gene expression following the most recent radiation exposure. Gecko screening confirms candidate genes' function by measuring stable cells' proliferation following radiation therapy using the CCK8 test. After digestion with EDTA, 1000 cells per well were injected onto 96-well plates, and the ideal radiation dosage was administered for various hours. The absorbance was measured at 37°C.

A 60 mm culture dish was used for inoculating the cells (Corning). After being attached to the wells, the cells were cultivated for 14 days after being irradiated at various doses (0, 2, 4, 6, 8, and l0Gy). Following removing the growth media, the cells underwent two PBS washes and a 15-minute methanol fixation. After discarding the fixative solution, a 0.1% concentration of crystal violet reagent was used to stain the material. Using a magnifying glass, the number of colonies with more than 50 cells was tallied. NanoDrop was used to identify mRNA accurately. ACTIN served as the internal control. The corresponding expression was computed using the $2-\Delta\Delta CT$ technique. A minimum of three instances of each data set were made. The Radio Immunoprecipitation Assay (RIPA) obtained protein concentration from culturing cells. The protein was then broken down using SDS-PAGE and subjected to antibody incubation. ImageJ

was used for the grayscale analysis that was performed. When expressing data, the mean ± standard deviation is used. At least three replications of each experiment were conducted. The statistical analyses specified for every experiment were the repeated-measures ANOVA and the independent sample t-test ($P < 0.05$).

3. ANALYSIS AND DISCUSSION

The C666-1 cell line's colony formation and CCK8 experiment were revealed. This indicates that the ideal irradiation dose for C666-1 cells was 2Gy/1f (Figure 1A-B). Following a 48-hour puromycin treatment at 3 ug/ml dosage, the cell-killing effectiveness was nearly 100%. As a result, 3 ug/ml was chosen as the study's working puromycin concentration (Fig. 1C). VIRUS CRISPR-Cas9-sgRNA library Screening the experiment's findings shows that, in gathered the cells, the successful gene will demonstrate some concentration seven days after irradiation and greater abundance fourteen days later (Fig. 1D). By integrating the clustering of differential genes with bioinformatics, 210 genes associated with radiosensitivity and radioresistance in non-pallid porcine cancer (NPC) were found, of which 131 were decreased and 79 had elevated levels (Fig. 1E, F). To comprehend the roles that the 210 genes that were chosen play in biological procedures, molecular activities, and cellular components, a GO investigation was performed on them (Fig. 1G).

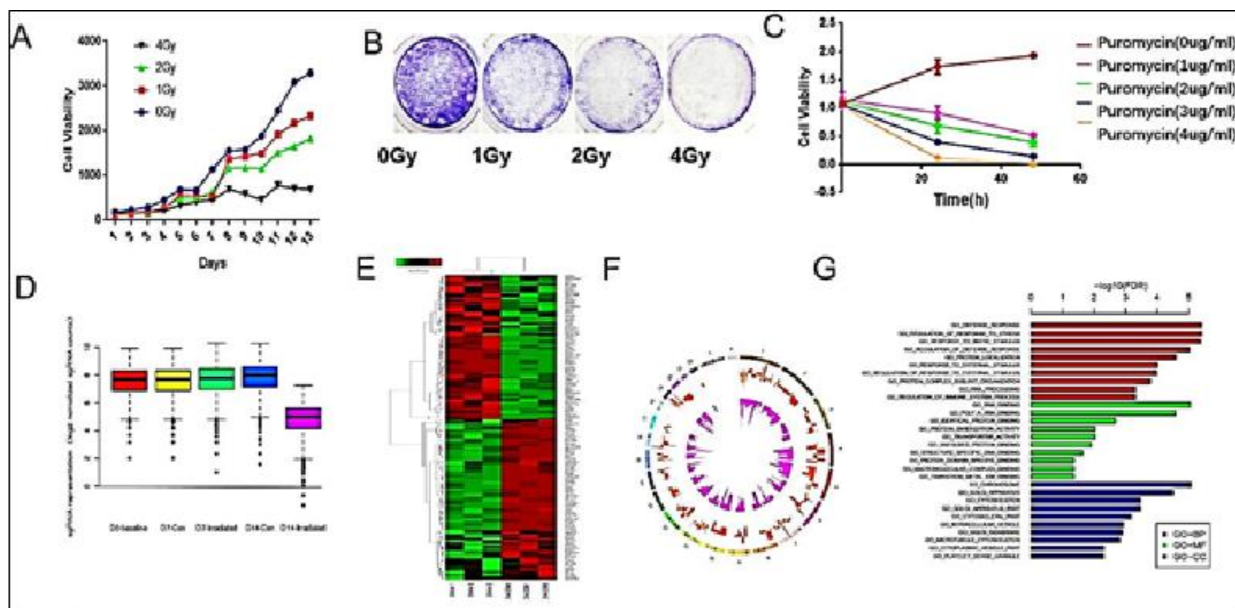

Figure 1 (A-G): Combining second-generation genome sequencing with CRISPR-Cas9 library lentivirus screening in radiation-treated nasopharyngeal cancer cells

Figure 2 (A-B) shows that the successful creation of NPC cell lines with a single essential gene (FBLN5, FAM3C, MUS81, DNAJC17, CALD1, TOMM20, CDKN2AIP, SNX22, PSIP1, SP1, TLN1) deletion was confirmed by real-time RT-PCR and WB. In Figure 3, in the CCK8 experiment and colony formation outcomes compared to the control group, the growth of C666-1 and CNE1 cells with low expression was significantly enhanced ($p < 0.05$). Figure 4 presented that C666-1R and 5-8FR cells grew more slowly and had a poorer capacity for colony formation than the control groups (Figure 4A–B), suggesting they were more radiation-resistant. Comparing C666-1R cells to C666-1 cells, the mRNA expression level of five genes was considerably reduced ($p < 0.05$) while the mRNA expression level of four genes was significantly increased ($p < 0.05$, Fig. 4C). Finding possible radiosensitive or radioresistant signalling pathways in NPC could be aided by GO and KEGG pathway analysis (Figure 5). The Fanconi anaemia

route and the TGF-beta signalling pathway are examples of the putative signalling pathways that KEGG enrichment analysis revealed may have contributed to radiosensitivity or radioresistance in NPC. In Figure 6 (A and B), the findings showed that radioresistant tumours had overexpressed SP1 and CDKN2AIP, whereas radiosensitive tumours had greater ($p < 0.05$) expressions of FAM3C, FBLN5, MUS81, and DNAJC17. The genome-wide CRISPR-Cas9 screening principle and the possible signalling pathway contributing to radiosensitivity or radioresistance in NPC are schematic.

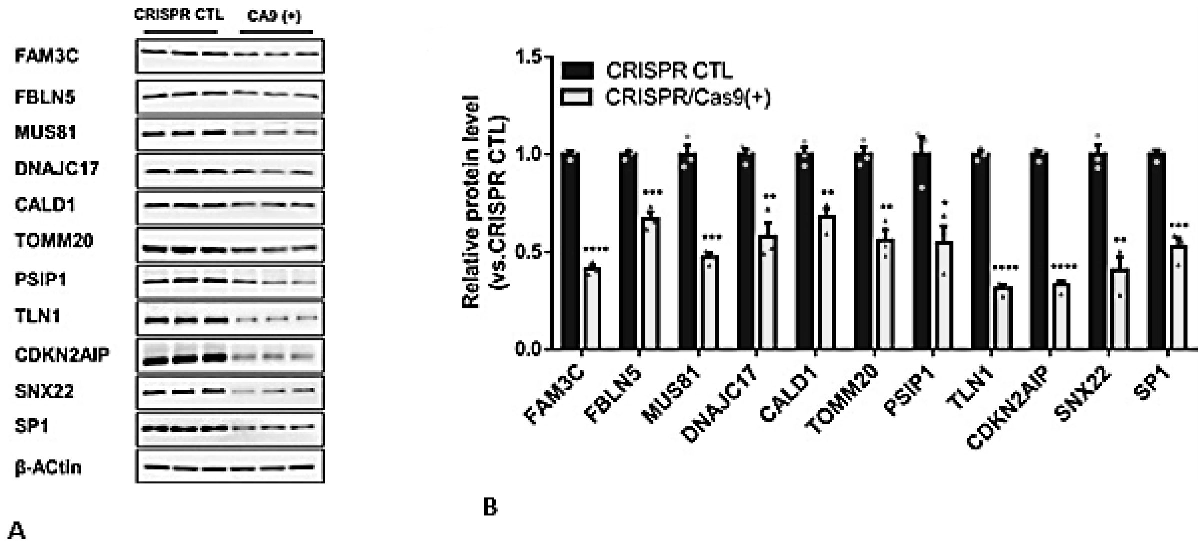

Figure 2 (A-B): CRISPR-Cas9-mediated knockdown efficiency of 11 vital functional genes in C666-1 cell.

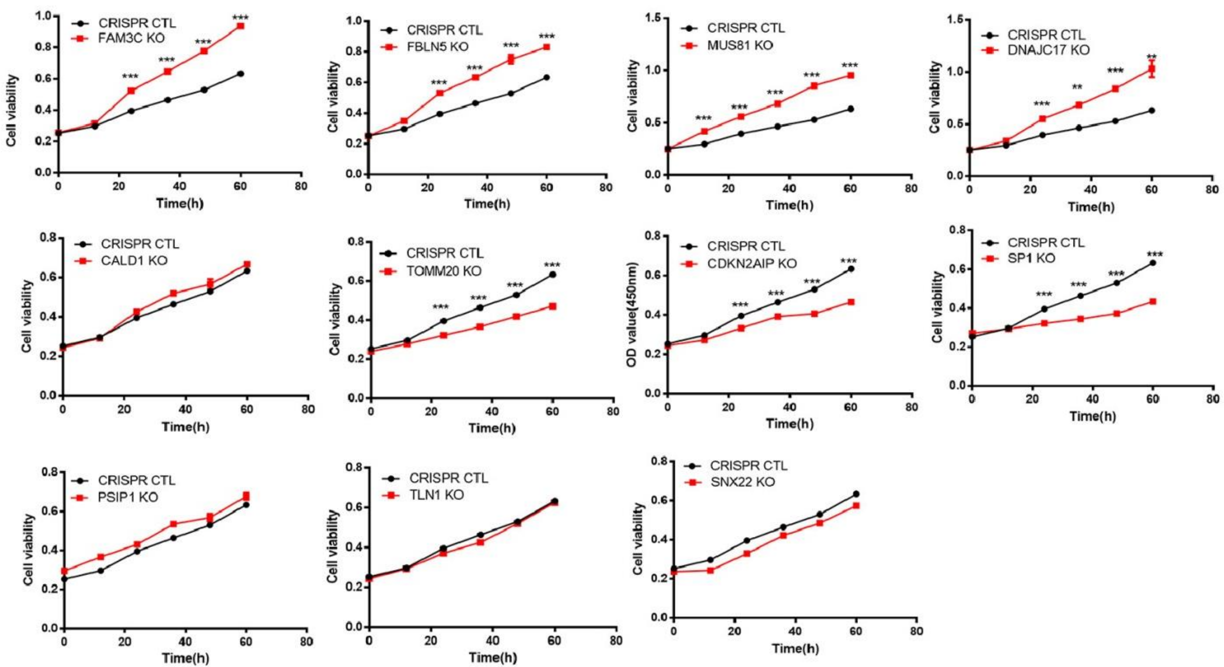

Figure 3: GeCKO screening results regarding the radiosensitivity and radioresistance of candidate genes in C666-1 cells

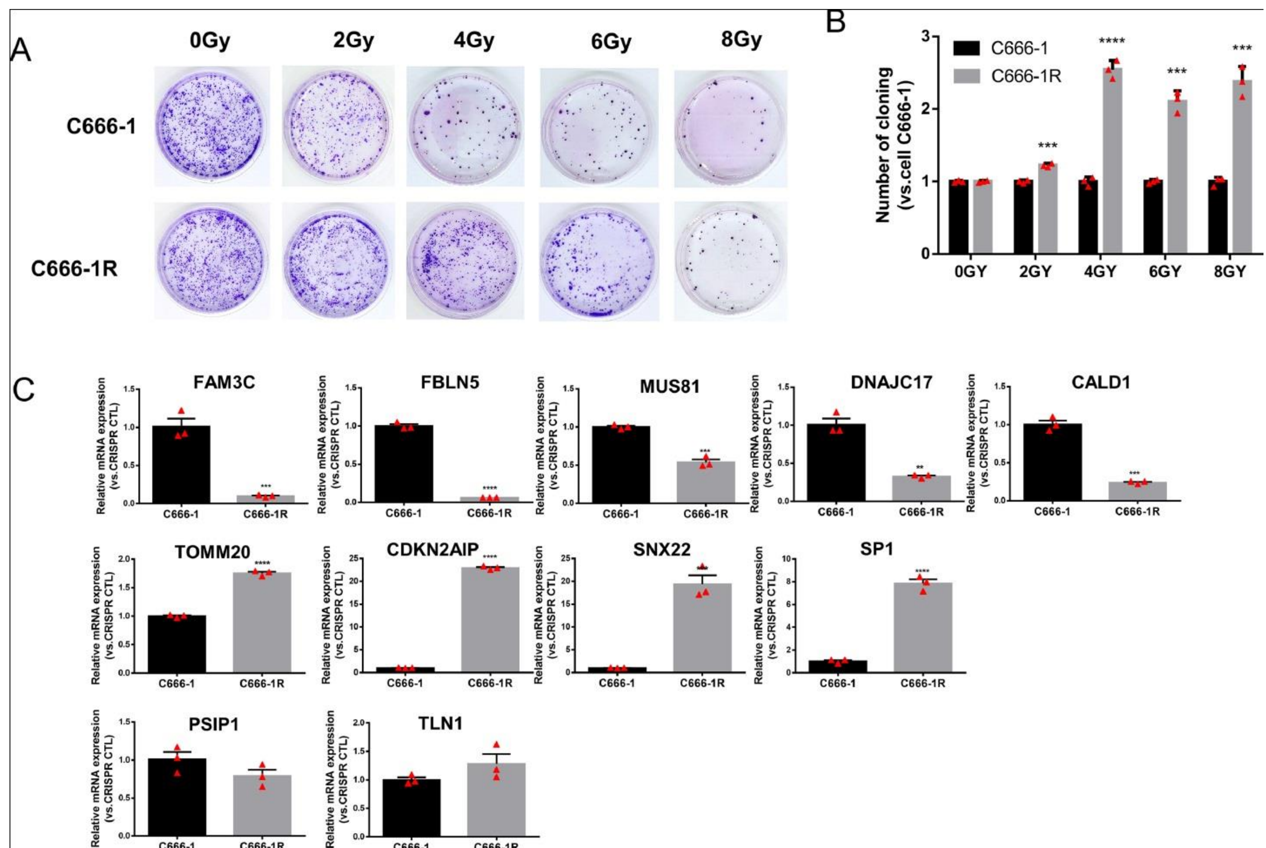

Figure 4 (A-C): The candidate genes' radiosensitivity and radioresistance were assessed using C666-1R. The results were the mean ± standard deviation from three different experiments. **** p < 0.0001, *** p < 0.001, and ** p < 0.01.

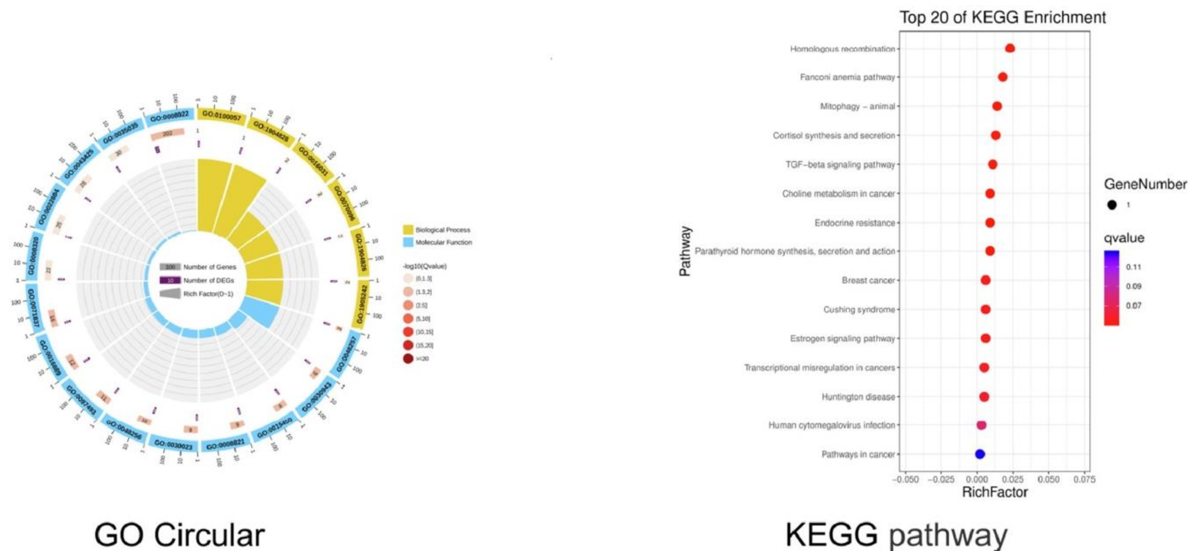

Figure 5: Go and KEGG pathway analysis for potential genes

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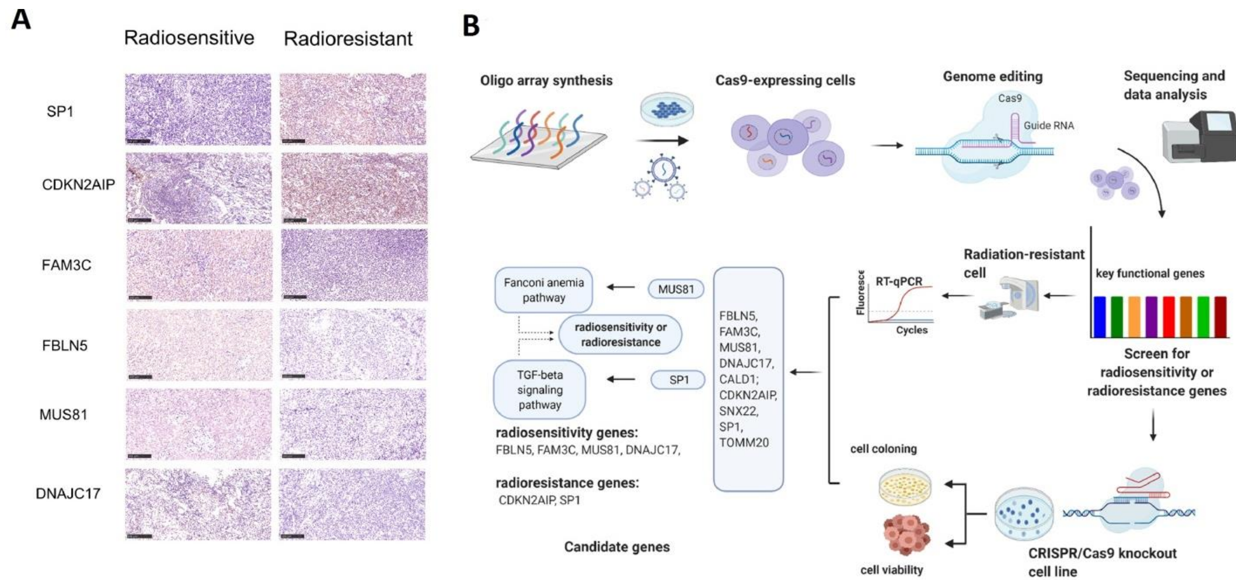

Figure 6 (A-B): Schematic illustrating the probable signaling mechanism

The foundation for enhancing the outlook of NPC patients is the screening of genes in NPC to increase the sensitivity of tumour cells. It is estimated that between 10% and 50% of the cells in solid tumours are hypoxia cells, which are 2.5–3.0 times more radioresistant than aerobic cells. They cause tumour recurrence and metastasis following therapy and cannot be eliminated [25]. High-throughput functional gene screening, which depends on gene editing technology, helps examine biological processes and evaluate gene function [26]. Furthermore, CRISPR-Cas9 depends on its benefits, which include a straightforward design, high specificity, high efficiency, and the capacity to generate various editing outcomes at the target location. Using CRISPR-Cas9 genome-wide screening, the researchers identified the target gene. They then narrowed down the gene range using pre-existing database analysis and performed a second screening in mice to identify the gene DCPS, which had a stronger correlation with the ability of AML cells to survive [27]. After analyzing transplanted tumour cells using genome sequencing and analytics, Shi et al. discovered 624 genes strongly linked to tumour metastasis [28]. According to this set of findings, the CRISPR-Cas9 genome-wide screening method may be crucial to the biological sciences. This research screened genes linked to radiosensitivity and radioresistance in NPC using the CRISPR-Cas9 genome-wide library lentivirus. Appropriate sgRNA are identified by second-generation sequencing technology. It is unknown; therefore, TLN1 and PSIP1 affect radiosensitivity and radioresistance. Of course, our work still has certain limitations, and additional research is required to understand the potential genes in NPC. MUS81 was first thought to have a radiation sensitization effect in NPC cells in this investigation. According to several findings, FA proteins may be crucial for maintaining genome stability during the cell cycle, particularly during the M phase [29], [30]. A transcription factor called SP1 binds to GC/GT-rich promoter regions to control the promoter activity of several genes related to cell cycle, differentiation, and carcinogenesis [31]. SP1 is a potential tumour suppressor gene [32], [33]. Several malignancies have overexpressed SP1, and so forth. The BMI1 gene promoter is synergistically bound by SP1 and C-MYC, which contributes to the pathophysiology of NPC [34]. According to Jun Wang et al. and our earlier study, SP1 is essential for raising the radioresistance of NPC cells. The radiosensitivity of NPC cells may rise when Sp1 is inhibited [21]. These reports align with the findings of the current investigation. As a result, SP1 is a viable therapeutic target and may be crucial in NPC's radiation resistance. CDKN2AIP, also called CARF or FLJ20036, has been demonstrated to be a newly discovered tumour inhibition regulator [35]. It is a novel protein that is elevated during carcinogenesis, stress-induced ageing, and replication. It causes dose-dependent apoptosis (downregulation) and senescence (overexpression) [36]. More reports need to be on

the involvement of DNAJC17 and CLAD1 in NPC formation.

4. CONCLUSION

Nasopharyngeal carcinoma (NPC) is a malignant tumor originating from epithelial cells in the nasopharyngeal cavity. It has a unique global distribution, with higher occurrence rates in Pakistan, Western Canada, Northern and Northeastern Africa, China, and Southeast Asia. Radiation therapy is the primary management option, but inherited characteristics, external factors, and exposure to EBV interact to cause the illness. External variables include alcohol intake, cigarettes, and nitrosamines. Genetic predisposition to EBV infection is linked to the disease. Radiation therapy, the primary management option for non-proliferative cancer (NPC) due to its high sensitivity to ionizing radiation (IR), causes cell death by triggering mortality signals through the production of reactive oxygen species (ROS), destruction of DNA, and stress responses. It is concluded that 9 genes were implicated in radioresistance and radio-sensitivity on nasopharyngeal carcinoma and demonstrated the potential of genome-scale screening with Cas9 by observing an elevated rate of finding verification and a high degree of uniformity between independent guide RNAs encoding genes. The study will also help to increase the effectiveness of therapy for NPC, genome-scale CRISPR-Cas9 knockout screening for genes may offer novel perspectives on the biological processes.

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