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Molecular mechanisms of radiation resistance in colorectal cancer: in silico identification of AURKA, BIRC5 and PLK1 proteins as potential biomarkers

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Running Head: Biomarkers identification in colorectal cancer.

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ABSTRACT

Purpose: The development of radiation resistance by tumor cells severely affects the survival of colorectal cancer patients. The aim of this work is to study the molecular mechanisms involved in the resistance to radiotherapy treatment in colorectal cancer and the identification of key genes as possible biomarkers.

Methods: Data mining was performed in PubMed with the keywords “colorectal neoplasms”, “radiotherapy” and “resistance”, generating a total of 242 articles in which a series of inclusion and exclusion criteria were applied to select the articles of interest. Then, an In-silico analysis of the selected genes was performed with the bioinformatic tools: GeneCodis, Metascape, KEGG, REACTOME, STRING, STITCH, CHEA3, DGIdb, CTD and GEPIA.

Results: Different mechanisms and genes involved in radiation resistance were described. These are related to evasion of apoptosis, cell cycle dysregulation, epithelial-mesenchymal transition and repair of DNA breaks, with the last one being the most relevant and influential. The In-silico study carried out with 21 genes involved in radiation resistance showed the implication of FoxO signalling and EGFR tyrosine kinase inhibitor resistance as the most enriched pathways. In addition, the study identified the key proteins AURKA, BIRC5 and PLK1, showing multiple interacting chemicals and drugs; such as tamoxifen, omacetaxine mepesuccinate and hydroxyzine pamoate, among others.

Conclusion: The identification of multiple transcription factors that regulate the expression of these key genes as well as the validation in patient samples where higher expression is observed in tumor patients, conserved across tumor stages I-IV, suggests their potential as possible biomarkers.

KEYWORDS: Colorectal cancer; radiotherapy; resistance; ionizing radiation; radiosensitivity; molecular marker.

Introduction

Colorectal cancer is one of the tumors that have the greatest impact globally. In Spain, it is the most diagnosed cancer in both sexes with 43,370 new cases and approximately 11,000 deaths per year due to mortality. The adenocarcinoma constitutes 90% of all colorectal tumors (SEOM 2019).

Radiotherapy plays a role in colorectal cancer. It has been shown that the protocol of chemoradiotherapy followed by surgery is, to date, the optimal approach to treat rectal cancer in stages II and III (locally advanced) (SEOM, 2019; ACS, 2023). The advantages provided by radiotherapy in this tumor are numerous, such as a reduction in tumor size and staging, decrease in locoregional recurrence, increase in overall survival, and an increased preservation rate of the anal sphincter. The latter is essential for a better quality of life for patients after surgery (George et al. 2019).

Neoplastic cells carry out various mechanisms to promote their survival. These cells emphasize evasion of apoptosis, deregulation of the cell cycle, and a failure of the DNA repair system to protect the genome´s integrity. The genesis of resistance to ionizing radiation by tumor cells leads to a poorer prognosis since radiotherapy is one of the fundamental pillars of treatment (Alamilla-Presuel et al. 2022). For this reason, it is imperative to identify the molecular pathways that activate tumor cells to become resistant to radiation, as well as substances or molecules capable of inducing sensitivity. In addition, knowing biomarkers of resistance to radiotherapy can help radiation oncology professionals to adopt a more individualized approach and reduce the morbidity caused by irradiation in healthy tissues (Sagkrioti et al. 2022). Current research focuses on identifying core genes/proteins with the aim of discovering new therapeutic targets, combating treatment resistance, and achieving better outcomes (Xie et al. 2020; Alamilla-Presuel et al. 2022).

The aim of this work is to study the molecular mechanisms involved in radiation resistance in colorectal cancer and identifying key genes as potential biomarkers.

Materials and methods

Data mining

A PubMed search was performed using the keywords “colorectal neoplasms”, “radiotherapy”, and “resistance”. This search generated a total of 265 articles, and the following inclusion and exclusion criteria were applied to them (Fig. 1A).

Inclusion criteria

Articles published from 2014 onwards were included. In total, 116 results were obtained.

Exclusion criteria

Animal studies, metastatic cancer (TxNxM1), non-adenocarcinoma cell type, studies on cancer stem cells, and those not related to resistance or sensitization to radiation were excluded. The concomitant use of chemoradiotherapy was also considered an exclusion criterion. Finally, 27 articles remained for analysis.

In-silico analysis

The genes involved in the resistance to radiation were functionally analyzed. Fig. 1B describes the stages followed in the analysis and the databases used. Cluster identification analysis with Metascape (http://metascape.org) (Zhou et al. 2019), gene ontology (GO) enrichment (GeneCodis) (https://genecodis.genyo.es/) (García-Moreno et al. 2021; 2022), and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways (GeneCodis) were performed (Kanehisa and Goto 2000; Kanehisa and Sato, 2020; Fu et al. 2022). A REACTOME study was conducted in the Reactome database (https://reactome.org/) (Fabregat et al. 2017). In addition, a protein-protein interaction study with the Search Tool for the Retrieval of Interacting Genes (STRING) database (https://string-db.org/) (version 11.5) (Szklarczyk et al. 2017; Sagkrioti et al. 2022) and with the STITCH database (http://stitch.embl.de/) (Szklarczyk et al. 2016) were performed. Then, a transcription factor regulatory network study was conducted with ChIP-X Enrichment Analysis v.3 (CHEA3) (https://maayanlab.cloud/chea3/) (Keenan et al. 2019), and the description and function of each transcription factor was obtained from Harmonizome 3.0. (https://maayanlab.cloud/Harmonizome/) (Rouillard et al. 2017). A drug-gene interaction study was conducted using the Drug Gene Interaction database (DGIdb) (http://www.dgidb.org) filter only to show approved drugs (Freshour et al. 2020; Wang et al. 2021). Curated chemical–gene interactions data were retrieved from the Comparative Toxicogenomics Database (CTD), (MDI Biological Laboratory, Salisbury Cove, Maine, and NC State University, Raleigh, North Carolina) (http://ctdbase.org/) (Davis et al. 2022). The Gene Expression Profiling Interactive Analysis (GEPIA) website (http://gepia.cancer-pku.cn/) was used to analyze RNA sequencing expression data in tumor and normal tissue, based on hundreds of patients from the GTEx projects and TCGA database (Tang et al. 2017). The hypergeometric statistical test was used with a *p*-value adjusted to obtain the most enriched annotations (Carmona-Saez et al. 2007; Nogales-Cadenas et al. 2009; Tabas-Madrid et al. 2012; Liu et al. 2023). The last access to these databases was in February 2024.

Results and discussion

The molecular mechanisms of resistance to radiation were evaluated, distinguishing the studies published in cell lines from the results observed in patients.

Radioresistance in colorectal cancer cell lines

Table 1 shows the results found in cell lines. The study published by Wang et al. (2017) concluded that the overexpression of BIRC5 (survivin), a molecule belonging to a family of inhibitors of apoptosis, via CXCL12/CXCR4 inhibits apoptosis and promotes cell survival. This same fact is observed in the studies of Ma et al. (2020) and Spagnoletti et al. (2018) who studied the underexpression of BTG3 induced by hypoxia and the presence of the BRAF gene mutation (BRAF V600E), respectively. lncRNAs are molecules that play a role in epigenetic suppression. In this way, Liu et al. (2020) and Xu et al. (2014) reported that the increase in the expression of LINC00630 and lncRNA R05532, NR_015441, and NR_033374 decrease apoptosis and increase cell survival after exposure to ionizing radiation.

Regarding radioresistance related to DNA strand breaks repair, Deng et al. (2018) elucidated a mechanism that involves overexpression of the CREB-KDM4B-STAT3 axis, KDM4B being a histone demethylase whose increase deregulates histone modifications, causing an improvement in the repair of genetic material. Along the same lines, RAD50 upregulation optimizes DNA repair, conferring resistance to radiation treatment, according to Chen et al. (2018).

The work by Kim et al. (2018) shows that the overexpression of the NDRG1 gene, related to N-myc, induces a reduced response to radiotherapy both through increased cell survival and DNA repair. miRNAs are non-coding RNAs that regulate gene expression by blocking mRNA translation. Specifically, miR-222 and miR-155 also cause an effect similar to that of NDRG1, through their interaction with PTEN and FOXO3a, respectively. This fact is reflected in the studies of Khoshinani et al. (2017) and Zhu et al. (2022).

The epithelial-mesenchymal transition (EMT) is a process that gives cancer cells properties similar to those of stem cells and greater metastatic capacity by reducing cell-cell adhesion. The overexpression of the OCT4 transcription factor influences this process and, therefore, results in a pathway of resistance to radiation, as concluded by Shao et al. (2018).

Some analyses were inconclusive. This was the case with Park et al. (2021) who unsuccessfully tried to delve into the cell death pathway induced by Ca^{2+} and the expression of CRMP4, which belongs to the family of cytosolic phosphoproteins. The results of Gnosa et al. (2014) regarding the presence of the AEG-1 oncogene were not significant either.

Radioresistance in samples from colorectal cancer patients

Various authors have obtained biopsies from patients with colorectal adenocarcinoma and, in some cases, from healthy tissues adjacent to the tumor. In these samples, markers that predicted the response to radiotherapy were studied. In addition, it was verified whether the radioresistance mechanisms previously analyzed in vitro also appeared in vivo (Table 2).

Wang et al. (2014) report that high levels of CEA are associated with a lower response to radiotherapy, mainly in terms of reducing the tumor stage. The study of Gnosa et al. (2014) relates the overexpression of the AEG-1 oncogene to a higher risk of distant tumor recurrence and lower disease-free survival in patients treated with radiotherapy. Lastly, Yao et al. (2014) investigated HER-2 as a marker of response to treatment without conclusive results.

Regarding the mechanisms of radioresistance that were previously analyzed in cultured cells, we found that overexpression of the CREB-KDM4B-STAT3 axis (Deng et al. 2018), epigenetic suppression of the BEX1 gene (Liu et al. 2020) and underexpression of BTG3 mediated by hypoxia (Ma et al. 2020), correlate with the results obtained in patient samples. In addition, comparing the samples of tumor tissue with those of normal tissue, a significant increase in the expression of the RAD50 gene was observed in neoplastic cells, being even greater in cells with greater resistance to radiotherapy. For this reason, Chen et al. (2018) proposed it as a radioresistance biomarker. In addition, Wu et al. (2014) found that IGF-1R overexpression is related to an increase in DNA repair and a decrease in apoptosis, all of which leads to greater resistance to treatment with ionizing radiation.

Radiosensitization in colorectal cancer

Table 3 shows published works, in cultured cells and patient samples, related to radiosensitizing molecules and/or molecular mechanisms involved.

Regarding radiosensitizers, Sahlberg et al. (2014) proposed the use of siRNA-PKcs that inhibit protein kinases related to non-homologous recombination of genetic material. Thus, the sensitivity to radiation is increased causing failures in DNA repair, affecting cell survival. The research by Shao et al. (2016) addresses the issue of the tumor stroma and its repercussion regarding treatment. After using rapamycin (antifungal), an induction of autophagy of TAMs was observed, which promotes angiogenesis and tumor growth among other effects. This induction resulted in an increase in apoptosis and an inhibition of cell proliferation after irradiation. Wang et al. (2017) studied the CXCL12/CXCR4 axis and proposed it as a radioresistance mechanism. Its inhibition with AMD3100 therefore increased cell death and radiosensitivity.

Abnormal cells with a mutated p53 gene cannot stop the cell cycle to permit DNA repair. An increase in cell apoptosis ranging from 53% to 80% was observed in mutated p53 cell lines when DHA was administered (Hosseini et al. 2018). Acriflavine is a molecule that has been found to sensitize cells to the action of chemotherapeutics. Lin et al. (2022) compared its efficacy in

radiotherapy, and found the activation of p53, causing mitochondrial dysfunction and, in turn, an increase in cell apoptosis.

PLK1 is a kinase that is involved in the cell cycle. Its inhibition by BI2536, according to Lund-Andersen et al. (2014), causes the cycle to stop in the G2/M phase, which turns out to be the most radiosensitive. In the study by Spagnoletti et al. (2018) numerous radiosensitizing agents were analyzed, mainly focused on cell lines with the mutated BRAF oncogene. First, both the BRAF inhibitor PLX4720 and the chemotherapeutic 5-fluorouracil (5-FU) were tested, with both results being inconclusive. However, silencing BRAF with an siRNA showed a very weak increase in apoptosis. By inhibiting CDK1, the cell cycle regulatory kinase, the rate of apoptosis was increased. Finally, the inhibition of HSP90 is correlated with decreased clonogenic potential and increased apoptosis (Spagnoletti et al. 2018).

As the last radiosensitization method investigated, we found the use of electroporation, which consists of applying high-voltage electrical pulses, thus increasing the permeability of the cell membrane. Yadollahpour et al. (2018) discovered that this method was synergistic with other radiosensitizers and that it also produced reactive oxygen species (ROS) in the membrane, damaging the cells.

Drake et al. (2015) highlight the fact that activation of the cellular stress response pathway to misfolded proteins can act as radiosensitizer. This pathway indirectly inhibits cell proliferation. Indeed, there appears to be a mechanism to increase the effectiveness of radiotherapy. In relation to lncRNA and miRNA, in the work of Zou et al. (2018), it has been described that if the lncRNA OIP5-AS1 is overexpressed, the expression of miR-369-3p is decreased, which upregulates DYRK1A, which is a kinase involved in cell proliferation. In this way, cell proliferation and viability decrease and apoptosis increases. Another miRNA involved in radiosensitization is miR-31 which, according to Zhang et al. (2022), when overexpressed, seems to decrease DNA repair by interacting with genes such as MLH1, decreasing cell survival.

The study by Kim et al. (2018) hypothesizes that downregulation of the NDRG1 gene sensitizes cells by disrupting DNA repair mechanisms, implying a reduction in cell proliferation and survival. A similar outcome occurs when the RAD50 gene is downregulated. An increase in apoptosis was observed with a decrease in cell survival after radiation from ~58.7% to ~35% in HCT116 cells and from ~62% to ~32% in HT29 cells, according to the analysis carried out by Chen et al. (2018).

Among the molecular pathways that increase radiosensitivity, and stop the cell cycle in the G2/M phase, we found the silencing of PSME3, and the inhibition of AURKA, a regulator of mitosis. These mechanisms have been studied by Song et al. (2019) and Liu et al. (2021), respectively.

The fractionated radiotherapy scheme at low doses can induce the EMT process in tumor cells, generating resistance. In this way, Kim et al. (2020) state that when SOCS1 is

overexpressed, the production of intracellular and mitochondrial ROS that arise after fractionated radiation is reduced, thus decreasing EMT and improving the radiosensitivity of the cell.

The article by Mhaidly et al. (2022) discusses the role of fibroblasts, cells that, when activated by the inflammatory interleukin IL1-α, transform into inflammatory fibroblasts, acquiring resistance to chemotherapy and radiotherapy.

In-silico analysis

The radiation resistance mechanisms found in the described studies, in cell lines and in patient biopsies, identified 21 proteins involved (Supplementary Table 1). The in-silico analysis allowed the identification of the core genes.

Identification of clusters

The gene interaction analysis performed showed a network where 13 clusters were identified, ordered by their statistical significance (hypergeometric test). Among them, the highest statistical probability was for the EGFR tyrosine kinase inhibitor resistance cluster ($p<1.91\times10^{-11}$), followed by extracellular vesicles in the crosstalk of cardiac cells ($p<1.26\times10^{-9}$), EGF/EGFR signaling pathway ($p<1.48\times10^{-9}$), androgen receptor network in prostate cancer ($p<1.45\times10^{-8}$), and regulation of the mitotic cell cycle ($p<5.62\times10^{-8}$), etc. (Supplementary Fig. 1).

GO enrichment analysis

The enrichment analysis showed the involvement of 13 biological processes, 13 cellular components, 9 molecular functions, and 25 KEGG pathways; all with a *p*-value <0.01 (hypergeometric test) (Fig. 2A,B). Protein phosphorylation and the negative regulation of the apoptotic process stood out with greater statistical significance as biological processes; the cell nucleus as the cellular component; identical protein binding as the most significant molecular function; and the KEGG pathways: FoxO signaling pathway, prostate cancer, and EGFR tyrosine kinase inhibitor resistance as the most enriched and significant pathways. The evaluation of the genes involved in this enrichment process showed that only 12 genes appear in the KEGG pathways analysis (Fig. 2C).

The REACTOME analysis showed that these genes were involved in developmental biology, immune system, cell cycle, programmed cell death, DNA repair, signal transduction, disease, metabolism of proteins, cellular responses to stimuli, and gene expression (transcription), with a *p*-value <0.05 (hypergeometric test) (Fig. 2D).

Protein–protein interaction analysis

The interaction analysis of the 12 proteins identified after GO enrichment showed their categorization into a single cluster related to gastric cancer (CL:4867) (false discovery rate: 0.0091). In this cluster, only the BIRC5, AURKA, and PLK1 proteins were found (Fig. 3A). The interaction analysis with STITCH identified the BIRC5 protein in the colorectal cancer (ID 05210) KEGG pathway. The three proteins in the STRING cluster showed multiple interactions among them in the STITCH analysis (Fig. 3B). These proteins constitute the core of all those previously described as related to radiation resistance in colorectal cancer.

Regulation mechanisms and interacting chemicals and drugs

Supplementary Fig. 2A shows the regulatory network of the 10 most significant transcription factors regulating the expression of the BIRC5, AURKA and PLK1 proteins, indicating of the greatest co-expression and co-occurrence by the thickness of the edges. It should be noted that the regulators E2F7, DRAP1, and ZNF511 were those with the highest integrated scaled rank (>0.999) (Supplementary Fig. 2B). The complete description of the most prominent transcription factors is included in Supplementary Table 2, noting that most of them are involved in some phenomenon related to the regulation of the cell cycle or cell proliferation.

Supplementary Fig. 3 shows the chemical agents that have the most interactions with these proteins, expressing the number of interactions with each one. In this way, the AURKA protein showed a greater number of interactions with bisphenol A, estradiol and benzo(a)pyrene (Supplementary Fig. 3A); the BIRC5 protein with arsenic trioxide, resveratrol and doxorubicin (Supplementary Fig. 3B); and the PLK1 protein with cisplatin, bisphenol A and benzo(a)pyrene (Supplementary Fig. 3C).

The search for drug–gene interactions resulted in the list shown in Supplementary Table 3. In this case, considering all the approved drugs, the AURKA protein showed interactions with 6 drugs, with the highest interaction score for tamoxifen. The BIRC5 protein showed interactions with 25 drugs. The highest score values were for omacetaxine mepesuccinate, plicamycin, oprelvekin, calcitonin, and irinotecan hydrochloride. Finally, the PLK1 protein showed interactions with 18 drugs, highlighting hydroxyzine pamoate with the highest score.

Despite the numerous chemical agents and drugs with which the AURKA, BIRC5, and PLK1 proteins interact, it is necessary to conduct efficacy and toxicity assays both *in vivo* and *in vitro*. The development of effective drugs targeting the identified proteins or their associated pathways may prove challenging. In this regard, the present work represents a preliminary screening approach that facilitates and guides subsequent necessary studies. The lack of pharmacological targets poses a significant challenge for the development of precision medicine. Moreover, existing inhibitory molecules may present unfavorable toxicity profiles, highlighting the need to continue searching for new, safer, and more effective molecules.

RNA sequencing expression data

To validate whether the core genes found could be considered potential biomarkers, the GEPIA website was used to analyze the expression levels in patients from the GTEx Project and TCGA database. The analysis of tumor samples from colon and rectum adenocarcinoma-affected patients showed hyperexpression of AURKA, BIRC5, and PLK1 proteins compared to the expression levels in normal colon and rectum control tissues (Fig. 4A–C) (*p*-value cutoff: 0.01). Furthermore, the study of the expression levels of these genes in tumors in the different stages (I–IV) showed that the average levels observed were similar for the three genes in all stages, with no statistically significant differences observed (Fig. 4D–F). These results highlight the importance of these genes/proteins in the focus of this work.

However, no study results have been found in patient samples correlating the expression levels of these genes with the response to radiation, beyond those described in this work through data mining. Therefore, further validation studies are needed to correlate the expression levels of AURKA, BIRC5, and PLK1, in colorectal cancer patients, with their response to radiotherapy. In this regard, the correlation between expression levels and radiation response in cell lines should be explored.

Although the validation results obtained from the GEPIA database highlight the increased expression of AURKA, BIRC5, and PLK1 in tumor patients, it is essential to emphasize that this observation alone does not constitute direct evidence of radiation resistance mechanisms. To strengthen this relationship, additional experimental data, such as functional assays and in vivo models, would be crucial to confirm the direct involvement of these transcription factors in mediating radiation resistance. This would help establish a direct causal link and solidify the potential of these proteins as therapeutic targets, thus contributing to a deeper understanding of the molecular pathways underlying treatment resistance.

Today, there is a wide variety of treatment modalities available to combat colorectal cancer, in some cases depending on the molecular target they address (Xie et al. 2020). Focusing on radiotherapy-treated tumors, patients are not free from recurrences, where the cells that repopulate the tumor are more resistant to treatment (ACS, 2023). In fact, cells that increase the DNA repair mechanisms have a higher rate of radioresistance. Numerous mechanisms have been described that induce resistance to ionizing radiation in multiple tumor types (Alamilla-Presuel et al. 2022). In colorectal cancer, the mechanisms are related to: apoptosis, cell cycle, DNA damage repair, and the epithelial-mesenchymal transition.

This study identified 21 genes involved in radiation resistance in colorectal cancer. The in-silico analysis revealed that the main implicated proteins are AURKA, BIRC5, and PLK1. These proteins showed multiple interactions with chemicals and drugs. The identification of multiple transcription factors that regulate the expression of these key genes, as well as the higher levels

of expression observed in tumors, which remain consistent across tumor stages I-IV, suggests their high potential as possible biomarkers.

Zhu et al. (2023) and Mukherjee et al. (2023) found an overexpression of AURKA protein in breast invasive carcinoma and in lung adenocarcinoma, respectively; suggesting this protein as a potential new prognostic biomarker and therapeutic target for these tumors. In addition, AURKA is overexpressed in a wide variety of cancer types including colorectal, where aberrant expression has been observed, linked to cellular resistance to drugs (Yang et al. 2023). These authors suggest that AURKA could be a predictive biomarker for various tumor types. In this way, García-Torralba et al. (2023) confirmed the predictive capacity of AURKA in a cohort of breast cancer patients. Inhibition of this protein enhanced radiosensitivity in breast cancer by promoting cell apoptosis (Li et al. 2023). Therefore, the AURKA protein plays an important role in regulating the cell cycle and cell division. In colorectal cancer, its overexpression is associated with tumor progression, invasion, and resistance to therapy; suggesting that it may be a prognostic biomarker and a possible therapeutic target.

BIRC5 plays a key role in cell division, interacting with caspases, and contributes to the regulation of apoptosis and mitotic cycle (Yu et al. 2023). This protein is highly expressed in colorectal cancer promoting cell proliferation, migration, and invasion. The down-regulation could suppress the promotion of colorectal cancer cells, through the regulation of immune cells (Guo et al. 2023). Moreover, BIRC5 is associated with chemotherapy resistance. Its inhibition sensitizes resistant cells to cytotoxic agents (Mirza, 2024). This protein plays a crucial role in the regulation of apoptosis and cell survival. Its overexpression has been associated with tumor progression, resistance to apoptosis and poor patient survival. It has been suggested that it may serve as a prognostic marker and a possible therapeutic target in treatment.

PLK1 was found to be overexpressed in different tumors. Its inhibition reduces cell proliferation and increases apoptosis. Zhang et al. (2023) suggest that PLK1 protein could be used as a possible therapeutic target and as a prognostic biomarker in lung adenocarcinoma. In addition, an improved oxaliplatin resistance was described by Yu et al. (2021) in colorectal cancer patients. This protein is involved in cell cycle regulation, especially in the progression of mitosis. Its overexpression is associated with tumor progression, invasion, and metastasis, as well as resistance to radiotherapy and worse patient prognosis. It is considered a potential therapeutic target.

Considering that colorectal cancer exhibits significant intratumoral and intertumoral heterogeneity, identifying universally applicable biomarkers presents a considerable challenge. Radiation resistance mechanisms may vary across different colorectal cancer subtypes, stages, and genetic backgrounds. In this regard, our work has focused on identifying potential markers related to resistance without considering the underlying mechanisms or tumor heterogeneity. One of the reasons for this approach has been the limited information obtained through data mining. For this reason, the study includes results from assays using patient samples and cell lines. An attempt to address this limitation is reflected in the expression results of AURKA, BIRC5, and

PLK1 obtained from samples of colorectal cancer patients at stages I-IV (Fig. 4D–F). Intra- and inter-tumoral heterogeneity highlights the need for future specific studies aimed at identifying markers for subtypes and stages. This work represents an initial approach to the problem through a global perspective that may facilitate future studies using patient samples or cell lines with the same genetic background.

The analysis of patient data obtained from the GEPIA database, indicates that multiple proteins are overexpressed in colon and rectal cancer compared to normal tissue. However, this dysregulation does not suggest that they can be used as molecular markers unless there is a clear correlation with treatment response and/or resistance. In this regard, it is crucial first to identify potential response markers and analyze the degree of correlation they have with tumors at different stages, subtypes, or genetic backgrounds. It is very likely that several potential markers could be individually correlated with treatment response, different tumor stages, and that combining multiple markers could provide more precise and robust information. Developing a predictive model based on the individual contribution of several markers to the overall analysis could be a valuable prediction tool, considering the association of each marker with treatment response and the biological characteristics of each patient's tumor. This model would help address the problem of intra- and inter-tumoral heterogeneity and bring us closer to personalized predictive medicine.

The phenomenon of acquired resistance to ionizing radiation exhibits a multifactorial and dynamic nature. It is multifactorial because numerous factors and mechanisms can induce resistance, as described in this work across multiple cell lines and patient samples. It is dynamic because the acquisition of resistance following repeated radiation exposures constitutes a process that may increase the level of resistance over time. This dynamic nature could occur during the course of a radiotherapy treatment, where the more resistant tumor cells could progressively adapt to the doses received. This circumstance may lead to treatment failure and the emergence of more resistant recurrences. This fact highlights the need to monitor the expression of resistance biomarkers over time. To address the phenomenon of radiation resistance acquisition, it is essential to understand the dynamic changes in protein expression as tumors respond to or relapse after radiotherapy.

The implementation of personalized medicine approaches based on biomarker analysis, including the need for efficient and cost-effective technologies for patient stratification and the selection of the most effective treatment, represents a significant challenge to address in the coming years. In this regard, in silico studies facilitate multiple screenings that streamline the focus of in vivo and in vitro studies, thereby reducing the number of assays and potential markers to analyze. Given the large number of tumor types, cellular subtypes, molecular factors, and genetic backgrounds, it is essential to avoid the obstacle of numerous laboratories, hospitals, or research centers searching for and analyzing markers without a well-defined common direction. In this context, creating research networks working towards the same goal would be highly effective, with the necessary financial support, of course.

The tumor microenvironment plays a crucial role in radiation resistance, as it interacts in a complex way with tumor cells, influencing their ability to survive treatment. The presence of factors such as fibroblasts, endothelial cells, and the extracellular matrix contribute to the alteration of the cancer cells' response to radiotherapy. These interactions not only affect the tumor cells' ability to repair radiation-induced damage but also modulate the expression and function of various biomarkers related to resistance. Therefore, it is essential to delve deeper into the study of the tumor microenvironment and its impact on the identified biomarkers in order to develop more precise therapeutic strategies that can address the problem of radiation resistance, thus improving treatment efficacy.

Conclusions

The study carried out has allowed the identification of the main mechanisms of radiation resistance in colorectal cancer, involving apoptosis, cell cycle progression, DNA damage repair, and the epithelial-mesenchymal transition. Twenty-one proteins have been identified that act through different molecular mechanisms and that, to a greater or lesser extent, induce resistance in cells that overexpress them. The in-silico study showed that AURKA, BIRC5 and PLK1 constitute the core proteins of that group. These proteins have interactions with multiple chemicals and drugs, such as tamoxifen, omacetaxine mepesuccinate, and hydroxyzine pamoate, among others. The identification of multiple transcription factors that regulate the expression of these key genes as well as the validation in patient samples where higher expression is observed, and conserved across tumor stages I-IV, suggests their potential as possible biomarkers.

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Declarations

Ethics statement

This article does not contain any studies with human participants or animals performed by any of the authors.

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CRediT authorship contribution statement

Marta Rodríguez-García: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Antonio M. Burgos-Molina:** Investigation, Data curation, Formal analysis. **Alejandro González-Vidal:** Investigation, Data curation, Formal análisis. **Francisco Sendra-Portero:** Investigation, Data curation, Formal analysis. **Manuel Bernal:** Investigation, Writing - review & editing. **Miguel J. Ruiz-Gómez:** Conceptualization, Supervision, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing - review & editing. All authors have read and agreed to the published version of the manuscript.

Declarations of competing interest

None.

Data availability

All data included in this study are available upon reasonable request by contacting the corresponding author.

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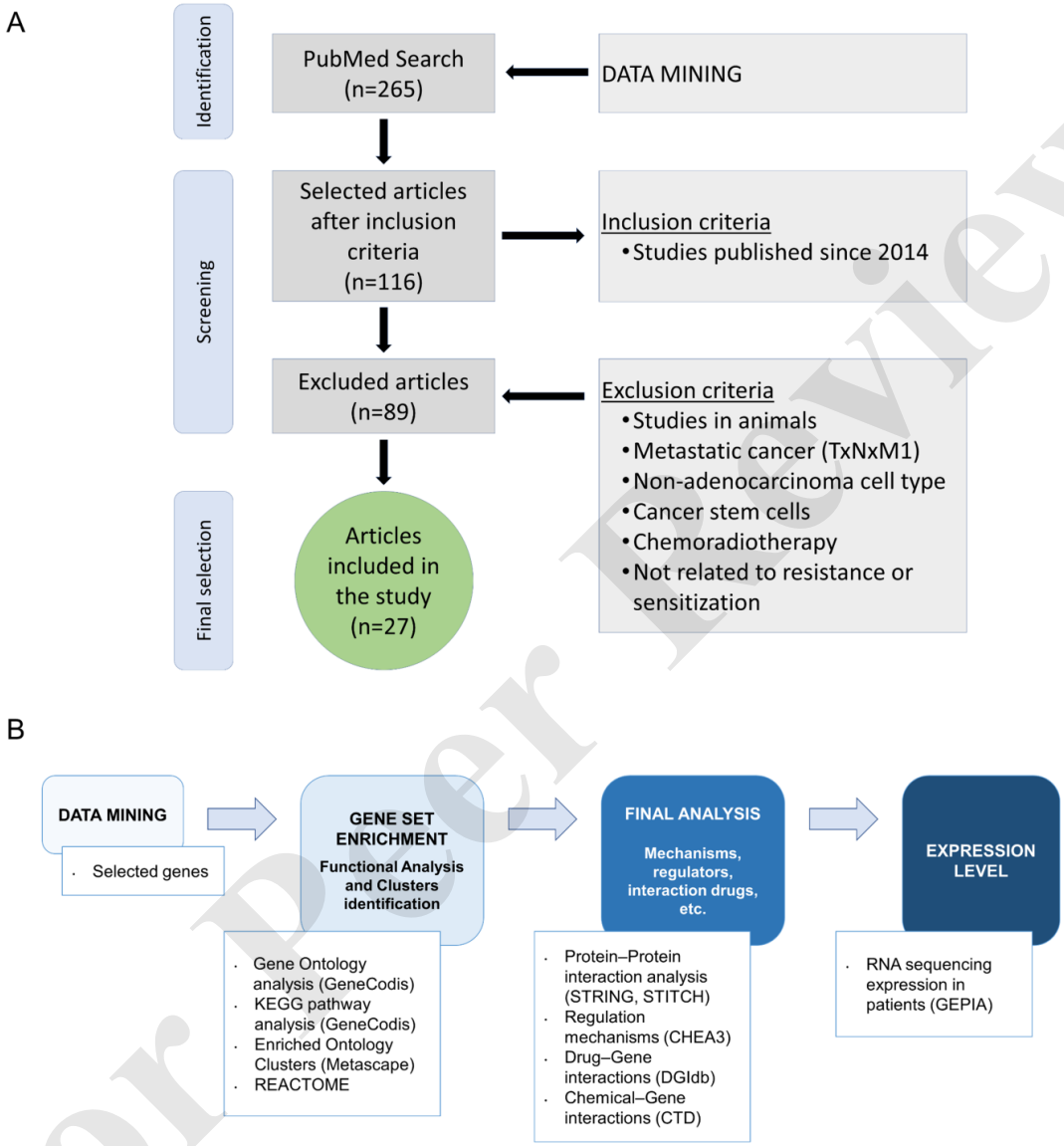
FIGURE LEGENDS

Figure 1. Methodology used in the study. **(A)** Flowchart of the strategy followed in the selection of articles. **(B)** Flowchart used in the In-Silico analysis. The stages followed and the databases used are indicated.

Figure 2. Gene set enrichment analysis. **(A)** GO enrichment for the genes found involved in radiation resistance. GO biological process, GO cellular component, and GO molecular function, $p<0.01$. **(B)** KEGG pathways, $p<0.01$. **(C)** Genes identified by KEGG pathways analysis. **(D)** Analysis of the Reactome, $p<0.05$. The hypergeometric test was used in all cases.

Figure 3. Protein–protein interactions. **(A)** Analysis of protein–protein interactions of genes identified by KEGG pathways. Local network cluster (CL, STRING). **(B)** Network proteins obtained by STITCH analysis, implicated in the colorectal cancer KEGG pathway.

Figure 4. Expression levels (RNAseq) of **(A)** AURKA, **(B)** BIRC5, and **(C)** PLK1 genes in tumor samples from patients with colon adenocarcinoma (COAD) and rectum adenocarcinoma (READ) (red boxplots), as well as in normal colon and rectum tissues (gray boxplots). Num(T): number of tumor patients. Num(N): number of normal patients. *p-value cutoff: 0.01. **(D, E, F)** Expression of AURKA, BIRC5, and PLK1 in tumor patient samples at different stages (I–IV). The analysis has been performed on the Gene Expression Profiling Interactive Analysis (GEPIA) website (http://gepia.cancer-pku.cn/).



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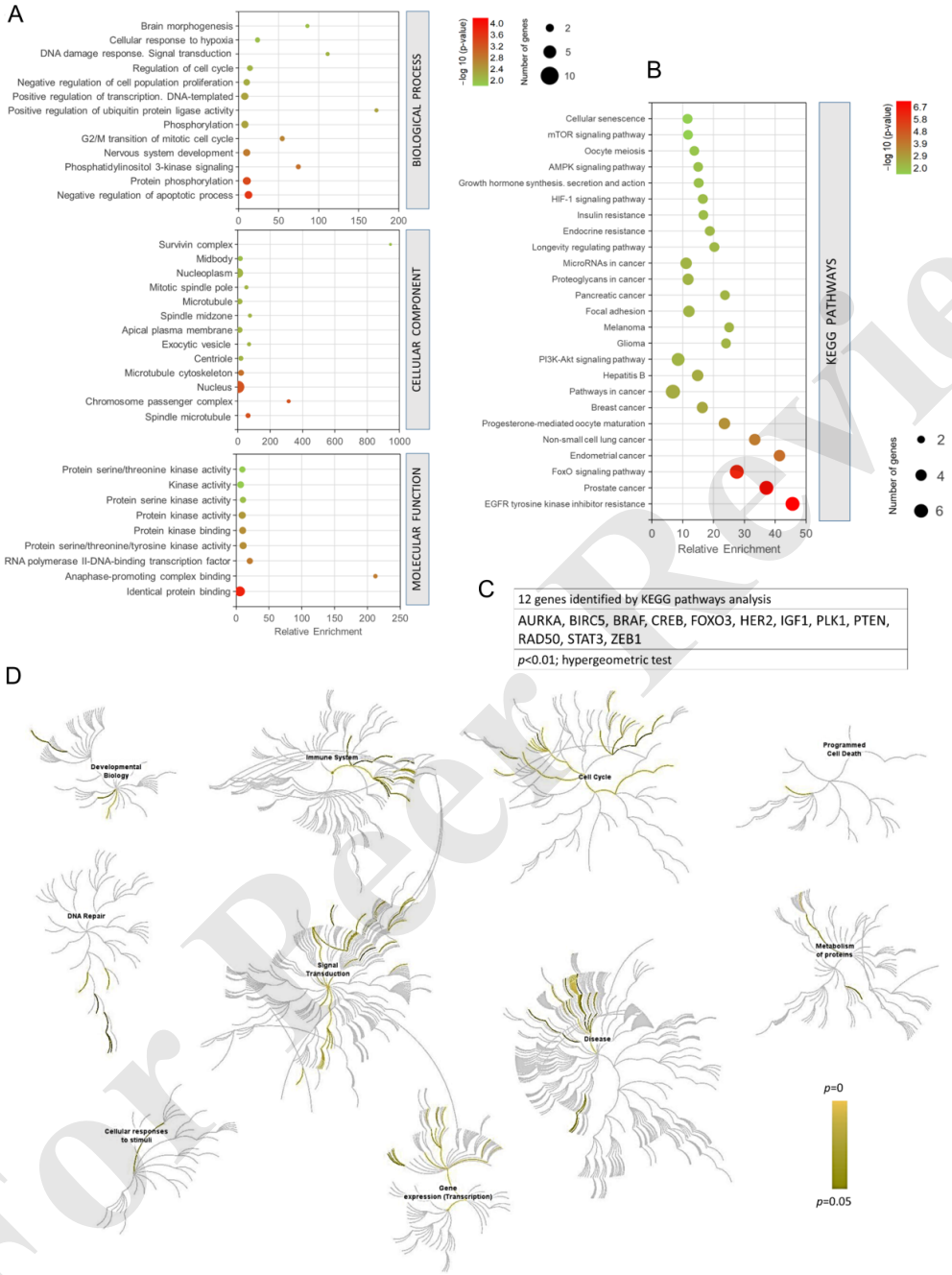

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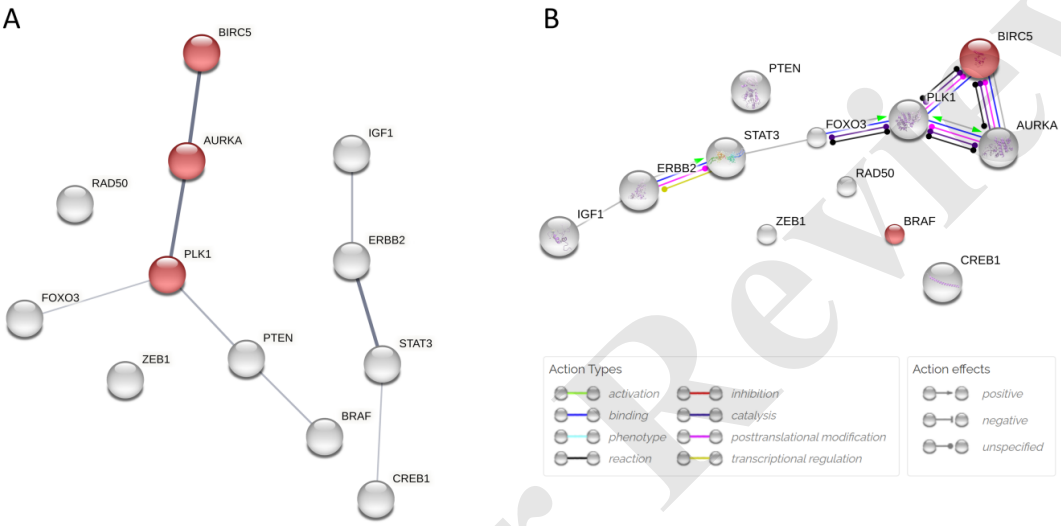

Cluster	Local network cluster (STRING)	Strength	False discovery rate (FDR)
CL:4867	Mixed, incl. mitotic cytokinesis, and Gastric cancer network 1	2.1	0.0091

Pathway ID	KEGG Pathway	False discovery rate (FDR)
05210	Colorectal cancer	0.00639

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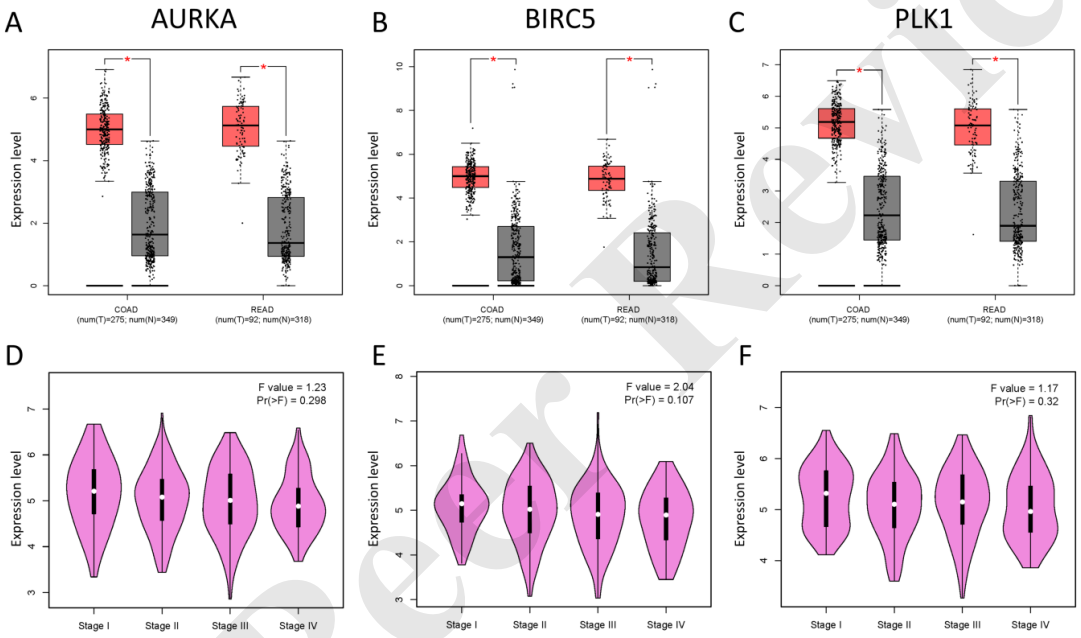

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Table 1. Articles that study radioresistance in colorectal cancer cell lines.

REFERENCE	CELL LINE	IRRADIATION CONDITIONS	MARKER / RADIORESISTANCE MECHANISM	EFFECT AFTER RADIATION
Gnosa et al., 2014	SW480, SW620, KM12C, KM12L4a, CCD-18Co, CCD-841 CoN	6 MeV, X-rays; 4.8 Gy/min	AEG-1	Inconclusive survival alteration
Xu et al., 2014	HT29, SW480, RKO, LoVo, HCT116	2 Gy	Overexpresion of lncRNA R05532, NR_015441 and NR_033374	83% increase in the surviving fraction
Wang et al., 2017	HCT116	1 Gy/min, gamma rays	Overexpression of survivin by activation of CXCL12/CXCR4	Increased cell survival Decreased apoptosis
Khoshinani et al., 2017	HCT116	6 MeV, X-rays; 200 cGy/min Total dose: 21-45 Gy	Underexpression of PTEN via PI3/Akt by overexpression of miR-222	Apoptosis inhibition Increased cell proliferation
			Underexpression of FOXO3a via PI3/Akt by overexpression of miR-155	Increased DNA repair Apoptosis inhibition Cell cycle disturbance
Chen et al., 2018	HCT116, HT29	2-4 Gy, gamma rays	Overregulation of RAD50	Increased DNA strand break repair
Spagnoletti et al., 2018	RAS/BRAF wild-type COLO320, KRAS G13D, HCT116, BRAF V600E, HT29	6 MeV, X-rays; 400 cGy/min	Disruption of BRAF V600E gene	Decreased apoptosis (79.4% surviving fraction in cells with mutated BRAF vs 35.7% in cells with wild-type BRAF)
Kim et al., 2018	SNU-61, SNU-283, SNU-503, SNU-977, SNU-977R80Gy, SNU-1411, SNU-1411R80Gy	Fractional irradiation. Total dose: 80Gy, gamma rays (40 cycles)	Overexpression of NDRG1	Increased DNA strand break repair Increased cell survival and proliferation
Shao et al., 2018	HT29 y SW480	0.61Gy/min, X-rays	Overexpression of OCT4/ZEB1	Increased epithelial-mesenchymal transition
Deng et al., 2018	HT29, SW620, CRL-1831, NCM-460	1Gy/min, gamma rays	Overexpression of CREB-KDM4B-STAT3	Increased DNA strand break repair
Liu et al., 2020	HCT116, SW480, LoVo, SW1116, LAS174T, FHC	2Gy/day, X-rays; total dose: 40Gy	Epigenetic deletion of the BEX1 gene via overexpression of LINC00630 (lncRNA)	Increased cell survival and viability Decreased apoptosis
Ma et al., 2020	CCD-18Co, SW480, HT-29	0.5 and 10 Gy, X-rays; 2 Gy/min	Hypoxia-mediated underexpression of BTG3	Increased cell survival Decreased apoptosis
Park et al., 2021	SW480, SW620, HT-29, DLD1, Caco2, LoVo, KM12C, KM12SM, RKO, HCT116, LS174T	5Gy, gamma rays; followed by 15 days recovery period. Total dose: 120Gy (24 cycles)	Relationship between the Ca^{2+}-induced cell death pathway and CRMP4 expression	Inconclusive

Table 2. Studies evaluating radioresistance in samples from patients with colorectal cancer.

REFERENCE	TYPE OF SAMPLE	RADIATION TREATMENT	MARKER / RADIORESISTANCE MECHANISM	EFFECT AFTER RADIATION
Wang et al., 2014	Patients (n= 240)	30Gy in 10 fractions	CEA	High pre-radiotherapy CEA levels are associated with a higher rate of early metastases and less post-radiotherapy downstage (TNM)
Yao et al., 2014	Surgical specimen of rectal adenocarcinoma (patients n= 142)	Not specified	HER-2	Inconclusive
Gnosa et al., 2014	Biopsies of rectal adenocarcinoma, non-adjacent normal mucosa, adjacent normal mucosa, and metastatic lymph nodes (patients n=158)	25Gy in 5 fractions in a median of 7 days	AEG-1	Increased risk of distant disease recurrence Shorter period of disease-free survival
Wu et al., 2014	Rectal adenocarcinoma biopsies (patients n= 87)	Dose rate of 2Gy/cycle doing a total of 25 cycles over a 5 week period, receiving a total dose of 50Gy	Overexpression of IGF-1R	Increased DNA strand damage repair Decreased apoptosis
Chen et al., 2018	Surgical specimens of colorectal cancer and biopsies of adjacent normal tissues (patients n= 80)	Total dose of 45-50.4Gy of radiation, for 5 weeks	RAD50	Significantly increased in tumor cells, and even more, in radioresistant cells
Deng et al., 2018	Biopsies of colorectal adenocarcinoma and peritumoral tissue (patients n= 20)	1Gy/min dose rate with Cs137	Overexpression of CREB-KDM4B-STAT3	Increased DNA strand damage repair
Liu et al., 2020	Biopsies of colorectal adenocarcinoma and adjacent normal mucosa (patients n= 50)	Dose rate of 2Gy/day from intermittent X-ray radiation, receiving a total dose of 40Gy	Epigenetic suppression of the BEX1 gene via overexpression of LINC00630 (lncRNA)	Decreased survival Decreased disease-free period Worst prognosis
Ma et al., 2020	Biopsies of colorectal adenocarcinoma (patients n= 24)	0, 5, and 10 Gy doses at a dose rate of 2 Gy/min with X-rays	Hypoxia-mediated BTG3 underexpression	Increased cell survival Decreased apoptosis

Table 3. Studies that address radiosensitizers and the molecular mechanisms involved in sensitivity to colorectal cancer radiotherapy.

REFERENCE	TYPE OF SAMPLE	RADIATION CONDITIONS	RADIOSENSITIVITY MECHANISM/AGENT	EFFECT AFTER RADIATION
Sahlberg et al., 2014	Cell lines: DLD1 and HCT116	1Gy/min dose rate with γ radiation	Inhibition of DNA repair proteins with siDNA-PKcs	Decreased cell survival
Lund-Andersen et al., 2014	Cell lines: HT29, SW620 and U2OS	Dose between 0-6Gy with X-rays	PLK1 inhibition with BI2536 inhibitor	Cell cycle arrest in G2/M
Drake et al., 2015	HT29 cell line Biopsies of colorectal cancer and adjacent normal tissue (patients n= 50)	2Gy every 7 days up to a cumulative dose of 22Gy with X-rays	Response to misfolded proteins (UPR)	Unknown mechanism. Unclear
Shao et al., 2016	Cell line: LoVo	Dose rate of 198 cGy/min with X-rays reaching doses of 6Gy	Rapamicina mechanism of action	Induction of autophagy of tumor-associated macrophages (TAMs) Increased apoptosis Inhibition of cell proliferation
Wang et al., 2017	Cell line: HCT116	Dose rate of 1 Gy/min with Co60	CXCL12/CXCR4 axis inhibition with AMD3100	Increased apoptosis and cell death
Chen et al., 2018	Cell lines: HCT116 and HT29 Surgical specimens of colorectal cancer and biopsies of adjacent normal tissues (patients n= 80)	2-4Gy of γ radiation (cells and tissues) Total dose of 45-50.4Gy of γ radiation over 5 weeks (pre-surgery dose)	RAD50 downregulation	Decreased DNA repair Decreased cell survival after radiation from ~58.7% to ~35% in HCT116 cells and from ~62% to ~32% in HT29 cells Increased apoptosis
Spagnoletti et al., 2018	Cell lines: RAS/BRAF wild-type COLO320, KRAS G13D HCT116 and BRAF V600E HT29	6MeV photons with linear accelerator at a dose rate of 400 cGy/min	BRAF inhibitors (PLX4720)	Inconclusive results
			Silencing of BRAF with a siRNA (siBRAF)	Very weak increase in apoptosis of BRAF-mutated tumor cells (BRAF V600E)
			5-fluorouracil (5-FU)	Inconclusive results
			CDK1 inhibition with RO3306	Increased apoptosis in colorectal tumor cells with BRAF mutation (BRAF V600E)
			Inhibition of the HSP90 chaperone with HSP990	CDK1 and BRAF underexpression Increased apoptosis in colorectal tumor cells with BRAF mutation (BRAF V600E) Inhibition of clonogenic potential in cells with BRAF mutated (BRAF V600E)
Zou et al., 2018	Cell lines: LoVo, SW480 and HEK293T	Dose rate of 2Gy/day with X-rays, receiving a total dose of 40Gy	Overexpression of lncRNA OIP5-AS1 upregulating DYRK1A via miR-369-3p	Decreased cell proliferation and viability Increased apoptosis
Kim et al., 2018	Cell lines: SNU-61, SNU-283, SNU-503, SNU-977, SNU-977R80Gy, SNU-1411 and SNU-1411R80Gy	Fractional irradiation of a total of 80Gy with Cs137 (40 cycles)	Disruption of DNA repair mechanisms through downregulation of NDRG1	Decreased cell survival and proliferation
Yadollahpour et al., 2018	Cell line: HT29	0, 2, 4, 6 and 8Gy doses with X-rays	Electroporation (1200 V/cm electrical pulse)	Synergistic effect with other radiosensitizers Production of reactive oxygen species (ROS) in the cell membrane
Hosseini et al., 2018	Cell line: HT-29 mutant for p53	2, 4, 6, 8 and 10Gy doses of γ radiation	Sensitization via survivin with 50 and 100μM docosahexaenoic acid (DHA)	Increased cell apoptosis (53% and 86%)

Song et al., 2019	Cell lines: Ls174-T, Caco-2, HCT116, HT29, SW620, SW480 and LoVo. Biopsies of colorectal cancer and adjacent normal mucosa both in paraffin embedding and fresh tissue (patients n= 163)	X-ray irradiation at different doses: 2, 4, 6 and 8Gy	CyclinB1 and CDK1 underexpression via PSME3 silencing	Cell cycle arrest in G2/M phase
Kim et al., 2020	Cell lines: HCT116, HCT116 p53 -/-, HT29, SW480 and SW620	Fractionated γ radiation of 2Gy/day for 3 consecutive days for a total dose of 6Gy	Action against epithelial-mesenchymal transition (EMT) via SOCS1	Decreased epithelial-mesenchymal transition (EMT) induced by ROS Increased cell apoptosis
Liu et al., 2021	Cell lines: HCT116, LoVo, SW480, RKO, Colo205 and HT-29 Colorectal cancer biopsies (patients n= 130)	4Gy for 24h	AURKA inhibition	Decreased cell growth Increased apoptosis Cell cycle arrest in G2/M phase
Zhang et al., 2022	Cell lines: HCT116 and LoVo	irradiation at different doses: 2, 4, 6 and 8Gy with X-rays for 7 days. Dose rate of 2Gy/day	miRNA-31 overexpression	Decreased cell survival Association with decreased DNA repair is proposed
Lin et al., 2022	Cell lines: CCT-18co and SW620 Biopsies of colorectal cancer and normal tissue adjacent to the tumor (patients n=unknown)	Not indicated	Acriflavine (ACF)	Mitochondrial dysfunction, increased apoptosis and decreased cell viability Activation of p53

Supplementary material

Molecular mechanisms of radiation resistance in colorectal cancer: in silico identification of AURKA, BIRC5 and PLK1 proteins as potential biomarkers

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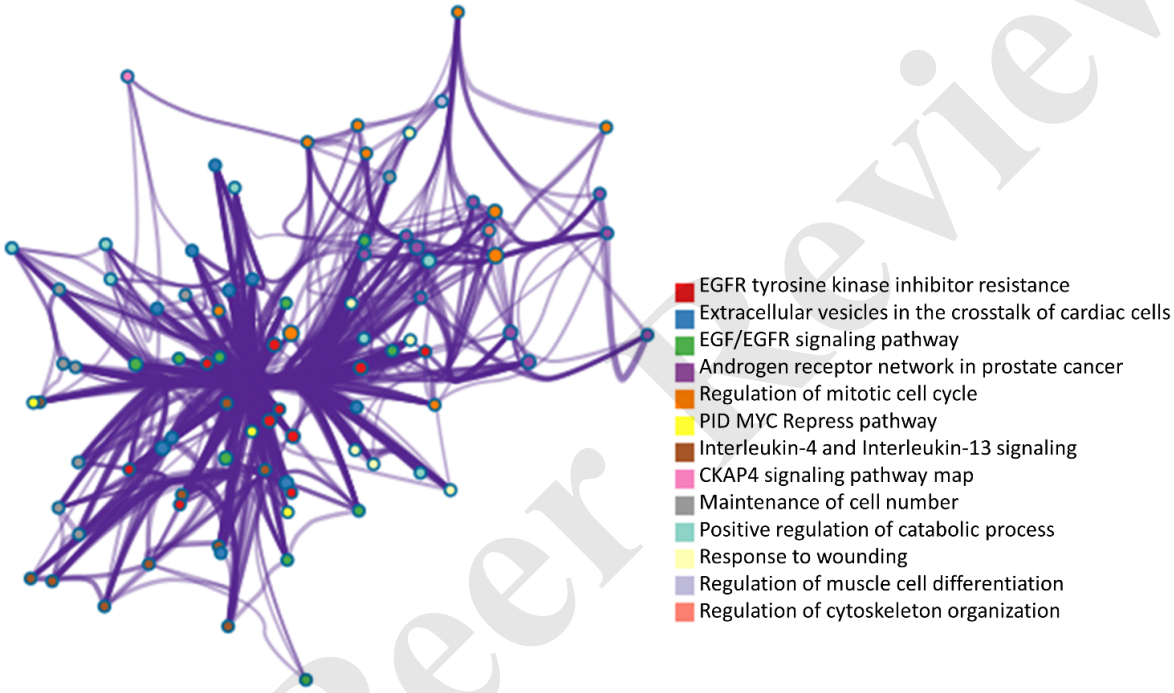
CONTENT

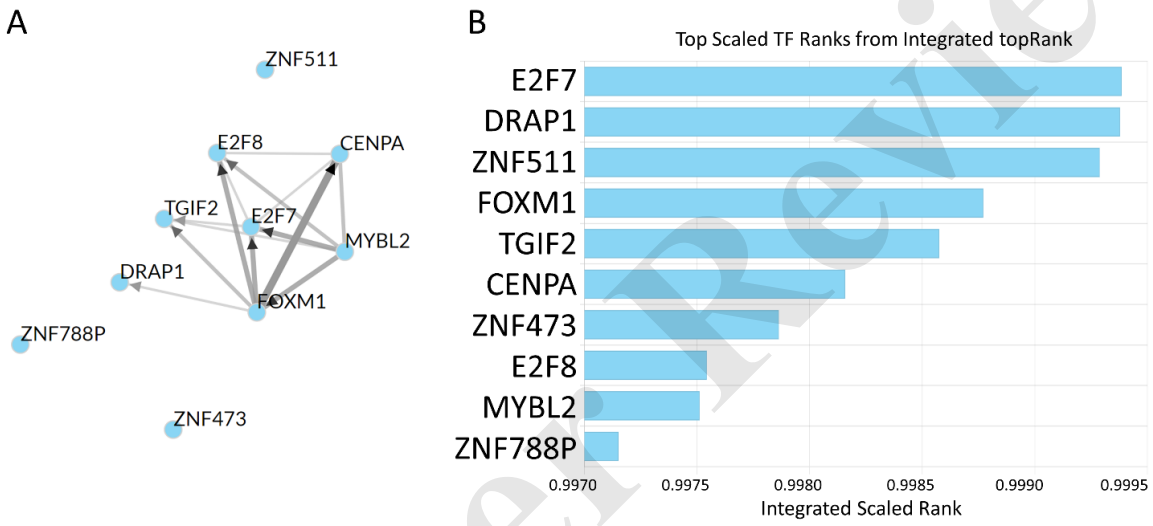
SUPPLEMENTARY FIGURES

Supplementary Fig. 1



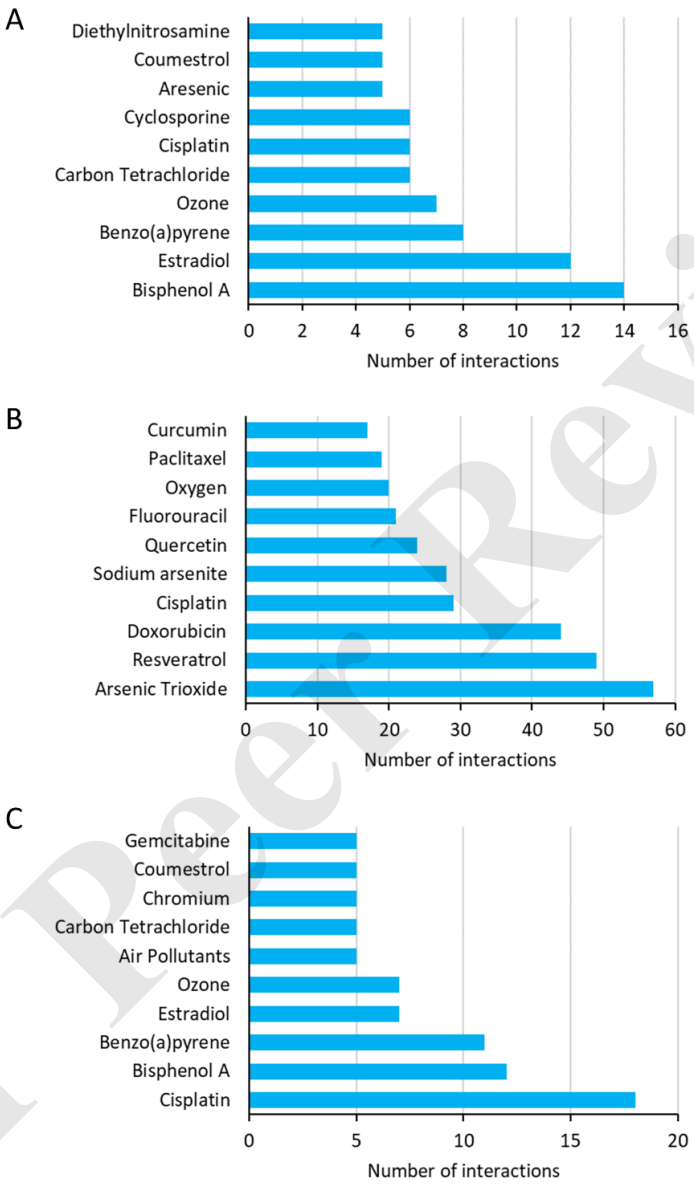
Supplementary Fig. 1. Network of interactions between genes and identification of clusters. Each cluster is shown by a color in the network. The interactions between clusters are linked by an edge. The greater the similarity between clusters, the greater the thickness of the edge.

Supplementary Fig. 2



Supplementary Fig. 2. Top integrated transcription factors that regulate the expression of the BIRC5, AURKA and PLK1 genes. **(A)** Transcription factors regulatory network. **(B)** Top scaled TF Ranks from integrated topRank.

Supplementary Fig. 3



Supplementary Fig. 3. Top interacting chemicals. **(A)** Chemicals that interact with AURKA protein. **(B)** Chemicals that interact with BIRC5 protein. **(C)** Chemicals that interact with PLK1 protein.

SUPPLEMENTARY TABLES

Supplementary Table 1

Gene	Description
AEG1	Metadherin
AURKA	Aurora kinase A
BEX1	Brain expressed X-linked 1
BRAF	B-Raf proto-oncogene, serine/threonine kinase
BTG3	BTG anti-proliferation factor 3
CEA (CEACAM7)	CEA cell adhesion molecule 7
CREB	cAMP responsive element binding protein 1
CRMP4 (DPYSL3)	Dihydropyrimidinase like 3
DYRK1A	Dual specificity tyrosine phosphorylation regulated kinase 1A
FOXO3	Forkhead box O3
HER2	Erb-b2 receptor tyrosine kinase 2
IGF1	Insulin like growth factor 1
KDM4B	Lysine demethylase 4B
NDRG1	N-myc downstream regulated 1
OCT4	POU class 5 homeobox 1
PLK1	Polo like kinase 1
PTEN	Phosphatase and tensin homolog
RAD50	RAD50 double strand break repair protein
STAT3	Signal transducer and activator of transcription 3
BIRC5 (Survivin)	Baculoviral IAP repeat containing 5
ZEB1	Zinc finger E-box binding homeobox 1

Supplementary Table 1. Genes involved in radiation resistance in colorectal cancer. Twenty-one genes obtained from studies in cultured cells and tumor patient biopsies.

Supplementary Table 2

Transcription factor	Description
E2F7	E2F transcription factors, such as E2F7, play an essential role in the regulation of cell cycle progression
DRAP1	DR1-associated protein 1 (negative cofactor 2 alpha). DR1 is a repressor that interacts with the TATA-binding protein (TBP) of TFIID and prevents the formation of an active transcription complex by precluding the entry of TFIIA and/or TFIIB into the preinitiation complex. The protein encoded by this gene is a corepressor of transcription that interacts with DR1 to enhance DR1-mediated repression. The interaction between this corepressor and DR1 is required for corepressor function and appears to stabilize the TBP-DR1-DNA complex
ZNF511	Zinc finger protein 511. Involved in deoxyribonucleoside metabolic process, ribosomal assembly and biogenesis
FOXM1	Forkhead box M1. Transcriptional activator involved in cell proliferation
TGIF2	TGFB-induced factor homeobox 2. DNA-binding homeobox protein and a transcriptional repressor, which appears to repress transcription by recruiting histone deacetylases to TGF beta-responsive genes
CENPA	Centromere protein A. Encodes a centromere protein which contains a histone H3 related histone fold domain that is required for targeting to the centromere
ZNF473	Zinc finger protein 473. Plays a role in histone 3'-end pre-mRNA processing and may be required for cell cycle progression to S phase
E2F8	E2F transcription factor 8. Regulates the expression of genes required for progression through the cell cycle. The encoded protein regulates progression from G1 to S phase by ensuring the nucleus divides at the proper time
MYBL2	V-myb avian myeloblastosis viral oncogene homolog-like 2. Nuclear protein involved in cell cycle progression. The encoded protein is phosphorylated by cyclin A/cyclin-dependent kinase 2 during the S-phase of the cell cycle and possesses both activator and repressor activities. It has been shown to activate the cell division cycle 2, cyclin D1, and insulin-like growth factor-binding protein 5 genes
ZNF788P	Zinc finger family member 788. Involved in regulation of posttranscriptional gene silencing and nuclear cell cycle DNA replication

Supplementary Table 2. Description of the top integrated transcription factors. These molecules regulate the expression of the BIRC5, AURKA and PLK1 genes.

Supplementary Table 3

Gene name					
AURKA		BIRC5		PLK1	
Drug	Interaction Score	Drug	Interaction Score	Drug	Interaction Score
Tamoxifen	0.05	Omacetaxine mepesuccinate	0.86	Hydroxyzine pamoate	0.04
Paclitaxel	0.03	Plicamycin	0.57	Topotecan hydrochloride	0.03
Fluorouracil	0.03	Oprelvekin	0.57	Cefaclor	0.03
Pazopanib	0.03	Calcitonin	0.57	Stavudine	0.03
Cisplatin	0.03	Irinotecan hydrochloride	0.49	Idarubicin hydrochloride	0.03
Sorafenib	0.02	Trastuzumab	0.33	Benzbromarone	0.02
		Sulindac	0.29	Acitretin	0.02
		Prasterone	0.19	Acriflavine	0.02
		Romidepsin	0.18	Inamrinone	0.02
		Flutamide	0.18	Oxytetracycline	0.02
		Tretinoin	0.18	Erythromycin	0.02
		Epoetin alfa	0.18	Thimerosal	0.02
		Lapatinib	0.13	Lansoprazole	0.01
		Epirubicin	0.11	Omeprazole	0.01
		Indomethacin	0.10	Hexachlorophene	0.01
		Vorinostat	0.07	Disulfiram	0.01
		Erlotinib	0.07	Simvastatin	0.01
		Paclitaxel	0.07	Dipyridamole	0.01
		Imatinib	0.05		
		Carboplatin	0.04		
		Dexamethasone	0.04		
		Doxorubicin	0.04		
		Fluorouracil	0.03		
		Methotrexate	0.03		
		Docetaxel	0.02		

Supplementary Table 3. Drug-gene interactions of the main genes found. Only approved drugs are shown. Search was performed in the Drug Gene Interaction database (DGIdb) (http://www.dgidb.org).