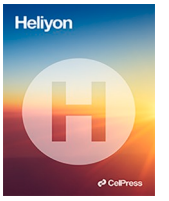
Heliyon 9 (2023) e21775

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



MiR-548c-3p through suppressing *Tyms* and *Abcg2* increases the sensitivity of colorectal cancer cells to 5-fluorouracil

Elham Khalili [a,b], Ali Afgar [c], Azam Rajabpour [b], Seyed Hamid Aghaee-Bakhtiari [a], Khadijeh Jamialahmadi [d,**], Ladan Teimoori-Toolabi [b,*]

[a] *Department of Medical Biotechnology and Nanotechnology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*
[b] *Molecular Medicine Department, Biotechnology Research Center, Pasteur Institute of Iran, Iran*
[c] *Research Center for Hydatid Disease in Iran, Kerman University of Medical Sciences, Kerman, Iran*
[d] *Biotechnology Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran*

A R T I C L E I N F O

Keywords:
Colorectal neoplasm
Drug resistance
5-Fluorouracil
TYMS protein
Human
ATP binding cassette transporter
Subfamily G
Member 2
MIRN548 microRNA

A B S T R A C T

Background: Colorectal cancer, is one of most prevalent the cancer in the world. 5-Fluorouracil is a standard chemotherapeutic drug while the acquisition of resistance to 5-Fluorouracil is one of the problems during treatment. In this study, we aimed to find the miRNAs that modulate the expression of *Tyms* and *Abcg2* as resistance-inducing genes in the resistant cell lines to 5-Fluorouracil.

Methods: 5-Fluorouracil-resistant HCT116 and SW480 cell lines were generated by consecutive treatment of cells with 5-Fluorouracil. This resistance induction was validated by MTT assays. The expression of the *Tyms* and *Abcg2* gene and miR-548c-3p were studied by quantitative real-time PCR in the cell lines.

Results: We hypothesized that miR-548c-3p is targeting *Tyms* and *Abcg2* simultaneously. Increased expression *Tyms* gene in the two most resistant cell lines derived from HCT116 and all resistant cell lines derived from SW480 except one were seen. Increased expression of *Abcg2* was observed in the most resistant HCT116-derived cell line and all resistant cell lines, derived from SW480. In all resistant cell lines, the expression of miR-548c-3p was decreased.

Conclusion: It can be concluded downregulation of miR548c-3p is in line with *Tyms* and *Abcg2* overexpression in resistant cell lines to 5-Fluorouracil.

1. Introduction

Colorectal cancer is the third most common type of cancer accounting for the second cause of death related to cancer. The incidence of colorectal cancer varies in different parts of the world while the highest incidence is observed in North America, Oceania, and Europe, especially Eastern European countries. In contrast, in Asia, Africa, and South America, the incidence is lower [1]. The survival rate depends on early detection whereas the 5-year survival rate in patients suffering from stage III is reduced to 20 % in comparison with that in patients suffering from stage II [2]. Despite advances in the different treatment modalities for this type of cancer, such as

* Corresponding author. Molecular Medicine Department, Pasteur Institute of Iran, Tehran, Iran
** Corresponding author. Department of Medical Biotechnology and Nanotechnology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.
E-mail addresses: jamialahmadikh@mums.ac.ir (K. Jamialahmadi), lteimoori@pasteur.ac.ir (L. Teimoori-Toolabi).

https://doi.org/10.1016/j.heliyon.2023.e21775
Received 7 February 2023; Received in revised form 21 October 2023; Accepted 27 October 2023
Available online 5 November 2023

monoclonal antibodies, and effective preventive screening measures, colorectal cancer is still one of the most common and deadly malignancies worldwide [3]. Therapeutic methods such as surgery, radiotherapy, and chemotherapy are standard modalities for these patients. Chemotherapy of CRC mostly includes the usage of 5-fluorouracil, leucovorin, capecitabine, and oxaliplatin though 5-fluorouracil is the backbone of chemotherapy [4]. Despite the widespread usage of this drug, the response rate to 5-Fluorouracil, in combination with other drugs or as a single drug, is estimated to be about 50 % and 10 %, respectively [5]which is linked to drug resistance.

Studies about drug resistance to 5-Fluorouracil have shown that many genes are involved in the pharmacokinetics and pharmacodynamics of this drug [6]. One of the key enzymes involved in 5-Fluorouracil metabolism is the thymidine synthase which level determines the drug sensitivity. The distributing level of methylenetetrahydrofolate and the high-level expression of this enzyme determines the intrinsic resistance to 5-Fluorouracil [7]. After the drug enters the cells, it is converted into various metabolites and each of these metabolites shows a distinct role in cell toxicity. One of these metabolites, namely fluorodeoxyuridine monophosphate (FDUMP), competes for uridine monophosphate (UMP) 's position for TS. Thereafter, the triple complex of TS-FdUMP-CH2THF will be formed instead of TS-dUMP-CH2THF leading to the inhibition of TS. As the only source of dTTP production is the TS enzyme, this will lead to an imbalance in the deoxy-nucleotide pool, particularly the dATP/dTTP ratio but also increases the UMP in the cells. The accumulation of dUTP in the cell during dTTP depletion enhances the merging of UTP into the DNA strands by DNA polymerase leading to the excision of DNA by uracil-DNA-glycosylase, and P53 accumulation in the cells and apoptosis. Another metabolite of 5-Fluorouracil or FUTP is incorporated into RNA strands disrupting the RNA processing and function [8–11].

Another important gene family that plays a role in resistance to different anticancer drugs such as 5-Fluorouracil is the family member of ATP-binding cassette (ABC) transporter superfamily including breast cancer resistance protein (BCRP or *Abcg2*), *P*-glycoprotein (*Abcb1*), and multidrug resistance-associated protein 1 (MRP1 or *Abcc*1)[12]. In humans, there are 48 members of this family [12]. ABC transporters use energy from ATP hydrolysis and transport of different materials such as vitamins, lipids, and sterols. In addition to the efflux of drugs and toxins [13]. *Abcg2* is an important member of the ABC transporter superfamily that is expressed highly in the placenta and blood-brain barrier protecting the fetus and the brain from harmful compounds [14]. The role of *Abcg2* in drug resistance in cancer cells was confirmed in the 1970s and it was shown that a variety of drugs, including methotrexate, mitoxantrone, flavopiridol, and 5-Fluorouracil are suitable substrates for *Abcg2* while high expression of *Abcg2* is significantly correlated with decreased potency of these drugs [15].

MiRNAs are small noncoding RNAs consisting of 19–24 nucleotides that function through direct binding to the 3′UTR of their target genes leading to inhibition of transcription or translation [16]. MiRNAs play roles in resistance to anti-cancer drugs by modulating different signaling pathways [16,17] which were first described by Calin et al. in CLL patients [18]. Also, miRNA-mediated drug resistance was also reported in other studies too [19]. For example, decreasing levels of miR-302c-5p, miR-3664–5p, and miR-129–5p through the increasing level of *Abcb1* bring about resistance to oxaliplatin in colorectal cancer cells [16]. In another study, miR-608 is restrained through induced gemcitabine chemo-resistance in pancreatic cancer cells [17].

Each chemotherapeutic drug performs its anti-cancer activity through a specific cellular pathway. One of the most important antitumor mechanisms of 5-Fluorouracil is through cell cycle arrest and apoptosis. MiR-21 [20] or miR-10b can cause evasion of cancerous cells from 5-Fluorouracil-induced apoptosis by down-regulating pro-apoptotic proteins [21]. On the other hand, miR-761 induces the sensitivity of colorectal cancer cells to 5-Fluorouracil [22]. MiR-548c–3P belongs to a large primate-specific gene family [23]. The overexpression of miR-548c-3p in osteosarcoma cells could inhibit the proliferation of tumor cells and promote their apoptosis [24]. MiR-548c-3p might be tumor suppressors of osteosarcoma, and their expression levels could be used as important reference indexes to evaluate the benign and malignant levels of osteosarcoma. Other studies indicated that miR-548c-3p inhibited tumor progression by suppressing the HIF1-mediated VEGF signaling pathway in papillary thyroid carcinoma [25]. It is also reported that miR-548c-3p inhibits glioma cell proliferation and migration by downregulating *c*-Myc [26]. In another research, it was revealed that miR-548c-3p suppresses human breast cancer cell growth and invasion [27].

In this study, we hypothesized that a subset of miRNAs may be associated with 5-Fluorouracil sensitivities by targeting *Tyms* and *Abcg2* genes which are involved in drug resistance. Therefore, miRNAs targeting *Tyms* and *Abcg2* simultaneously were predicted by in silico methods. In the next step, 5-Fluorouracil resistant colorectal cancer cells derived from HCT116 and SW480 were generated and in these cell lines, the level of *Tyms* and *Abcg2* in addition to their targeting miRNAs were investigated.

2. Materials and methods

2.1. Cell culture

SW480 cell line was purchased from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Iran). The HCT116 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). HCT116 and SW480 Cell lines were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Life Technology, MA, USA) supplemented with 10 % heat-inactivated Fetal Bovine Serum (FBS), L-glutamine (2 mM), 100 units/mL of penicillin, and 100 μg/ml of streptomycin (Life Technology, MA, USA). The cells was cultured in an incubator at 37 °C in 5 % CO2. The culture media of cells were changed every three days. Before any procedure, the mycoplasma-free status of these cell lines was confirmed by molecular methods.

2.2. Establishment of 5-fluorouracil-resistant HCT116 and SW480 cell lines

To generate drug-induced resistant sub-lines from HCT116 and SW480 cell lines, they were gradually exposed to increasing doses

of 5-Fluorouracil. Both of these cell lines were from the same passage number of cell lines. For this purpose, the proper inhibitory concentration of 5-Fluorouracil was determined before each step. Thereafter, the cell lines were treated with 80 % of primary inhibitory concentration (IC50) for two weeks. During this process, the drug-contained medium in each flask was refreshed every 3 days for 2 weeks. After the treatment period, the drug-contained-medium was replaced with the medium lacking 5-Fluorouracil and enriched with L-glutamine (2 mM) and non-essential amino acids (0.1 mM) (GIBCO, USA) for 4–5 weeks. The remaining cells were propagated and their sensitivity to 5-Fluorouracil was studied by MTT assay. According to their new IC50, they were treated with 80 % of their IC50, and according to the above-mentioned protocol, the next round of resistance induction was done. The drug was diluted to 1/10,000 and 50mg/pml was used as the main stock of the drug. Doses used for resistance induction and making HCT116-5FU1, HCT116-5FU2, and HCT116-5FU3 were 1.752 ng/μl, 7.51 ng/μl, and 12.464 ng/μl respectively which were calculated from 80 % of the IC50s of their parental cell lines.

Doses used for resistance induction and making SW480–5FU1, SW480–5FU2, SW480–5FU3, SW480–5FU4, and SW480–5FU5 were 3.584 ng/μl, 10.144 ng/μl, 15.56 ng/μl, 21.608 ng/μl, and 27.832 which were calculated from 80 % of the IC50s from their corresponding parental cell lines.

The resistant cell lines that undergo the resistance induction in each step were from the second to third passages of cells. These steps were repeated until no viable cell remained after 2 weeks of treatment of cells with 5-Fluorouracil which lasted three and five times for HCT116 and SW480-derived cell lines respectively. HCT116-derived cell lines after each round of resistance induction were named HCT116-5FU1, HCT116-5FU2, and HCT116-5FU3. SW480-derived cell lines after each round of resistance induction were named SW480–5FU1, SW480–5FU2, SW480–5FU3, SW480–5FU4, and SW480–5FU5. This method has been used in previous studies too [16, 17].

2.3. Cell viability assay by MTT

MTT assay was performed to calculate the IC50 of cell in response to 5-Fluorouracilby using (4, 5-dimethylthiazol-2-Yl)-2, 5-diphenylte-trazolium bromide (Sigma, Germany). In each well of a 96-well plate, about 1×10^4 SW480 and HCT116 cells were seeded. After 24 h of incubation in a CO_2 incubator, the cells were treated with different concentrations of 5-Fluorouracil (10nM–1000μM) in triplicates. After 48 h of incubation, the media of cells were removed and they were treated with 10 μl of MTT solution. After 5 h, 100 μL of isopropanol solvent were added to each well and mixed gently on a shaker for 20 min. The light absorption of each well at 570 nm was measured by ELISA reader and then to attain accurate IC50 of 5-Fluorouracil, the toxicity curves for different concentrations of the 5-Fluorouracil based on the measured OD from MTT assay, were drawn by Graphpad software.

In all experiments, the same number of cells that were incubated with cell culture media without 5-FU were considered controls for the normalization of data. In each experiment, at least three well were used as controls. Wells without cells were used for blanking the optical density of treated wells.

2.4. Predicting targeting miRNAs

To select the best miRNAs that target 3′ UTR of *Abcg2* and *Tyms* genes simultaneously, different online tools are based on special algorithms such as seed match between mRNA-miRNAs, thermodynamic features of miRNA-mRNA duplexes, and cross-species conservation of the sequence were exploited. To reduce false positive results, they were rechecked manually. In this regard, in order to determine the exact location of miR-548c-3p on 3′ UTR of these genes, after using the https://genome.ucsc.edu/ site, the number of locations and the location of miR-548c-3p were determined on the chromosome. To match mRNA, all the isoforms of the studied genes were downloaded from www.ncbi.nlm.hih.gov, and miR-548c-3p was blasted to them by the Megablast option of ncbi.nlm.nih.gov/blast. The exact locations where miR-548c–3P is going to be linked were determined too.

These online databases in this study were as follows: miRbase (http://www.mirbase.org/ftp.shtml), targetscan (http://www.targetscan.org/), mirDB (http://www.mirdb.org/miRDB/download.html), miRBases (http://mirtarbase.mbc.nctu.edu.tw/), miRWalk (http://mirwalk.umm.uni-heidelberg.de/), diana (http://diana.imis.athena-innovation.gr/). In this study, we used prediction tools that are based on different algorithms. Scores were obtained from all prediction tools separately and these scores were summed up. Finally, total scores were calculated. Also, the distance of the targeted region in the 3′ UTR of the genes to the coding region of genes and the number of targeted regions in the 3′ UTR of genes determined our decision on selecting these miRNAs. To ensure and increase the accuracy of the selected miRNA in addition to the above-mentioned bioinformatics, tools Molecular Evolutionary Genetics Analysis (Mega software, Kumar S, Stecher G, LiM, Knyaz C, and Tamura K, 2018) was also used. This software has been designed to evaluate the alignment of sequences between nucleotides and proteins, as well as alignment between different genomes, draw a phylogenetic tree, estimate the rate of molecular evolution, and confirm the evolutionary hypotheses. Also, the interaction of this miRNA and 3′ UTR of *Tyms* and *Abcg2* was checked by RNAhybrid. It is a tool for finding the minimum free energy hybridization of long and short RNAs. The tool is primarily a means for microRNA target prediction. Also, to find the number of binding sites of miRNA to 3′ UTR of studied genes, the locations on the chromosome and to identify the number of nucleotides and the exact sequence of these regions, the UCSC site was used.

In this study, we used http://cm.jefferson.edu/rna22v1.0/ and https://bio.tools/rnahybrid to check the prediction of parameters since these two sites use Gibbs free energy changes (ΔG). Gibbs' free energy changes are calculated using enthalpy changes (ΔH) and entropy changes (ΔS) of a system. A reaction is considered thermodynamically possible when the energy change is negative or the energy of the system decreases as a result of that reaction. On the other hand, it means that the system becomes more stable after the decline in energy. In this case, the enthalpy changes in the system will be negative. This is the formula of enthalpy in regard to the free

energy and temperature.

$\Delta G = \Delta H - T\Delta S$

2.5. Reverse transcription and real-time Q-PCR

2.5.1. Stem-loop and primers design

To design a method for detecting the miRNA levels, stem loops were designed according to the previous studies [28]. In this method, the reverse primer and probe were universal. To increase the PCR efficiency, Tm was decreased, and the additional structure of the stem-loop deletion was enhanced. Complementary nucleotides to the miRNA region were attached to this structure so the stem loop could identify the target miRNA with high accuracy. To design primers for quantifying the number of transcripts, all mRNA isoforms of *Tyms* and *Abcg2* were retrieved from https://genome.ucsc.edu/ and www.ncbi.nlm.nih.gov. Then temperature, the secondary structures, and alignment between the primers and template of the designed primers were checked by the primer blast and gene runner programs.

2.5.2. RNA isolation and cDNA synthesis

About 2.5×10^6 of each cell line were seeded in each well of a six-well plate 24 h before RNA extraction. Total RNA was extracted from cells using RNX-plus (CinnaGen, Tehran, Iran) according to the previously mentioned protocols [29,30]. MiRNA was extracted with a similar protocol except for incubation of isopropanol elutes at −20 °C overnight and afterward centrifugation at 12,000 rpm at 4 °C. The resulting RNA purity was measured by Nanodrop spectrophotometer (NanoDrop™ One Microvolume UV–Vis Spectrophotometers) at 260 nm and 280 nm. Samples with a ratio of 260/280 of 1.8–2 were considered pure.

The cDNA synthesis mixture from miRNA was performed by the following method: 2 μg of extracted miRNA and stem-loop primers (50 nM) in the tube reactions were mixed and then pre-heated to 95 °C for 10 min. Immediately the tubes were transferred on the ice. Thereafter, 1 mM DTT (0.1 M, Bio basic, Canada), 1 mM dNTP (10 mM, CinnaGen, Tehran, Iran), 0.5 μl of RNase inhibitor (40U/μl, Thermoscientific, MA, USA) and 1 μl of RevertAid (200U/μl, Thermoscientific, MA, USA) enzyme were added and were incubated at 42 °C for 60 min leading to final incubation at 70 °C for 10 min. The synthesis steps of cDNA from mRNA are similar to the synthesis steps of cDNA from miRNA except for the usage of 0.5 μl of oligoDT instead of stem-loop primers. All cDNA synthesis reactions were done in PeqSTAR96*X* Universal thermocycler (Germany).

2.5.3. Quantitative real-time PCR

To assess the expression level of different genes at the mRNA level, the quantitative Real-Time PCR method was performed in StepOne-Plus™Real-TimePCR System. The final mixture for this study was composed of 5 μl of Master Mix Green High ROX™ (Ampliqon, Denmark), 1 μl of cDNA, and 0.5 pmol of each primer in the final volume of 10 μl. The resulting mixture was preheated at 95 °C for 15 min proceeding to 40 cycles of 95 °C for 30s, 60 °C for 30s, and 72 °C for 30s. Also, to ensure the specificity of the amplified parts, the melting curve was checked and its correctness was confirmed. All experiments were done in triplicates. For studying the expression of miRNA, the mixture is composed of 5 μl of Taqman master mix (Ampliqon, Denmark) and 0.2 μM of universal Taqman probe [28], 1 μl of cDNA and 0.5 μl of each primer (5 pmol) in the final volume of 10 μl. It was preheated to 95 °C for 15 min; proceeding to 50 cycles of denaturation (95 °C for 30s), annealing (58 °C for 30s), and extension (72 °C for 30s). All designed primers (Table 1) were synthesized by Metabion Company (Germany). All experiments were done in triplicates. For normalization, U47 was studied. Probe and primer sequences are given in the previous studies [16,28].

To plot the relative expression and fold change expression of mRNAs or miRNA, CT was calculated as the mean CT of replicates. ΔCTs were calculated as the CT of the studied gene in comparison with the CT of the housekeeping gene (*Gapdh* for mRNAs and U47 for miRNA). ΔΔCTs were calculated by subtracting ΔCT of studied samples from ΔCT of non-treated ones. As the efficiency of PCR was calculated as 95–100 %, the fold change of expression was calculated by the $2^{-\Delta\Delta CT}$ formula by raising the power of 2 to –ΔΔCT.

2.6. MiR-548c-3p transfection in SW480, HCT116 cell lines by calcium phosphate method

The calcium phosphate method was used for the transfection of cells as it was used in the previous studies [16,17,28]. One day

Table 1
Sequence of primers and stem loop.

Name of the gene	Sequence of oligonucleotides (5′–3′)
TYMS-F	TCAGTACATATTGCGCACATC
TYMS-R	TCTGGGTTTCGCTGAAGCTG
ABCG2-F	CACGATATGGATACGGCTTTG
ABCG2-R	CGATGCCCTCTTTACCAA
miR-548c-3p-F	AGCTAAATCTCAATTCTTTTGC
Universal reverse primer	ACACTCAGAGTCACTA
miR-548c-3p stem loop	CAATAGCTACACCTGTCACGGTAGTGGCCCTGCGTCCTGTAGTCTAATTGGCAAA

before transfection, 300 × 10^3 cells were transferred into each well of a 6-well plate. When the cells reached the necessary density (50–60 %), medium of the cells was replaced with fresh medium containing 10 % FBS. Thereafter, 20 μg of the miR-548c-3p precursor coding plasmid, and 16 ml of 1 % TE buffer, were added to the final volume of 422 μl of HEPES buffered water and mixed. Thereafter, 500 ml of HB2X solution was added drop by drop over a period of 2 min to the mixture and after vortexing, it was kept at room temperature for 20 min. Finally, the above mixture was slowly added dropwise. The plate was incubated for 4 h in a 37 °C incubator containing 5 % CO_2, and then the medium of the plate was replaced with the fresh medium. After 24 h, the cell culture medium was replaced with the fresh medium. Transfection efficiency in both cell lines was estimated to be 60–70 % by calculating the GFP expressing cells among all cells 48 h after transfection (data not shown).

2.7. Statistical methods

All Data obtained from the triplicate experiments were expressed as Mean ± Standard Error of Mean (SEM) using Graph Pad Prism version 8.0.2 (GraphPad Software, La Jol-la, CA, USA) and figures were drawn by this program. ANOVA test was used for statistical analysis. In addition, correlation studies were performed by Pearson correlation analysis if data were distributed normally. The Spearman test was applied to study the association between the level of miR-548c-3p and the level of expression of the *Tyms* gene in all cell lines since the data did not have a normal distribution. *P*-values≤0.05 were defined as significant. All experimental tests were done in triplicate.

3. Results

3.1. Different microRNAs targeting Tyms/Abcg2 were predicted

The specificity and uniqueness of the miRNAs targeting 3′UTR of *Tyms* and *Abcg2* genes were investigated by the bioinformatics approach. According to the above-mentioned criteria, miRNAs targeting *Abcg2* and *Tyms* simultaneously were studied and among them, miR-548c-3p was selected. This miRNA was predicted for targeting *Tyms* and *Abcg2* by Diana Tools, miRWalk, Target scan, mirDB, and miRbase tools. A brief representation for output of these programs is given in Fig. 1. Also, it is predicted to target 3′UTR of *Tyms* at 4 regions and 3′UTR of *Abcg2* at 7 regions. The seed region of this miRNA for *Tyms* consists of 6 nucleotides while the seed region for *Abcg2* consists of 7 nucleotides. Also, the minimum free energy of hybridization between miR-548c-3P and 3′UTR of *Tyms* was predicted to be −14.3 kcal/mol. The minimum free energy hybridization of between miR-548c-3P and 3′UTR of *Abcg2* was predicted to be −14/9 kcal/mol (Fig. 2A, B, and 2C). All these predictions were done by bioinformatics methods.

3.2. SW480 and HCT116 acquired resistance to 5-fluorouracil during 5 and 4 rounds of resistance induction

The resistant cell lines were generated by consecutive treatment of cells with 5-Fluorouracil. While the IC50 of the HCT116 cell line was 2.19, the IC50s of HCT116-5FU1, HCT116-5FU2, and HCT116-5FU3 were 9.34, 15.58, and 24.69 respectively. The IC50s of all

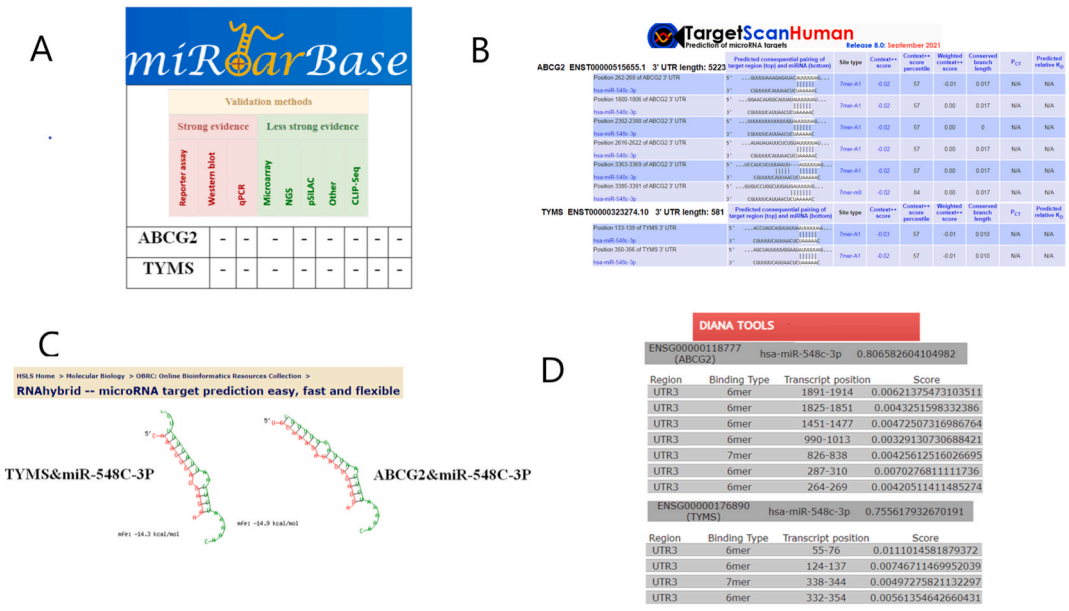


Fig. 1. The output of some online tools for prediction of binding sites for miR-548c-3p on 3′UTR of *Tyms* and *Abcg2* by A; miRbase, B: Target Scan, C: RNA hybrid, and D: DIANA Tools.

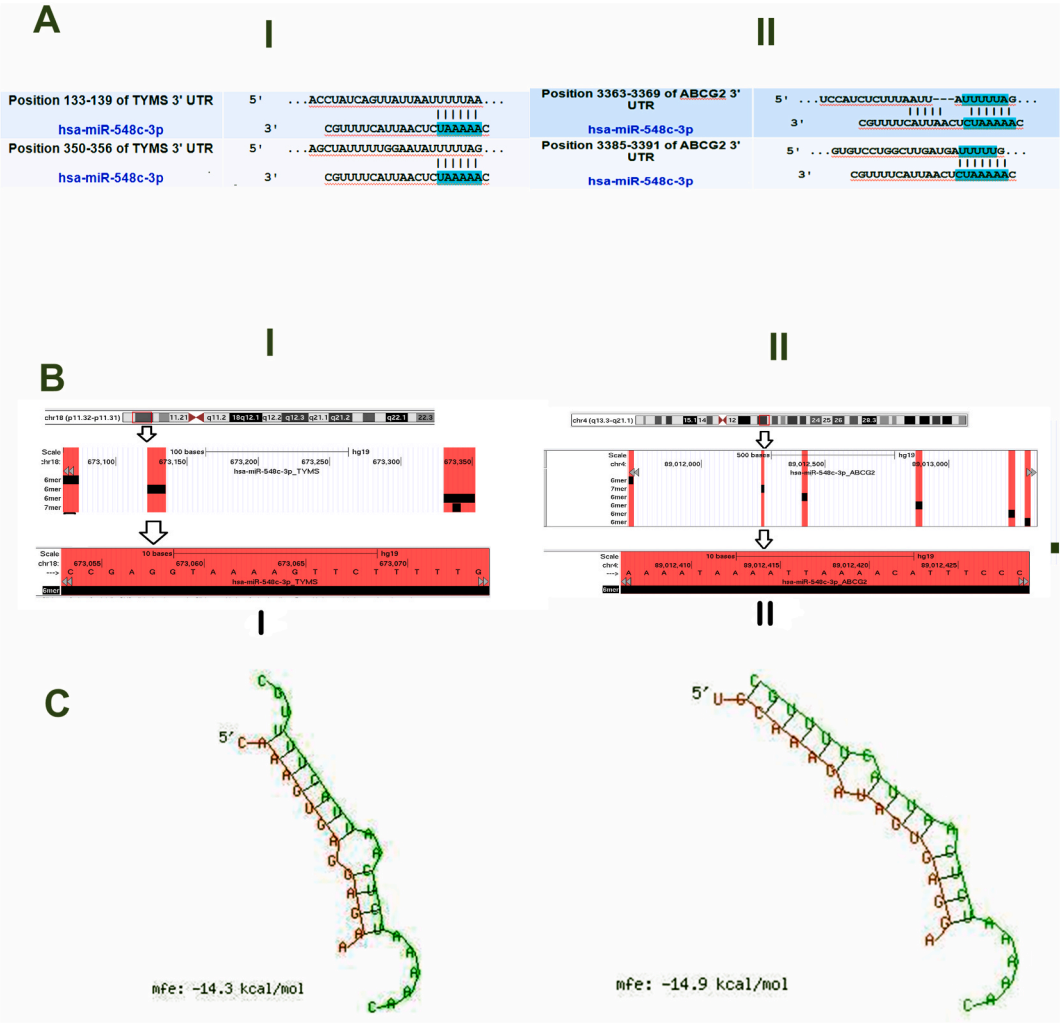


Fig. 2. A; Representation of miRNA-complementary sites located in 3'non-coding region of **AI**; *Tyms* transcript, and **AII**; *Abcg2* seed regions are highlighted in blue, **BI**; Representation of four sites of miRNA-complementary located on positions (673,053–673,074), (673,122–673,135), (673,336–673,342), and (673,330–673,352) of chromosome 18. Numbers are representative of chromosome location. One of these seed regions is highlighted and magnified below. Also, the seed regions of this miRNA are 6, 6, 6, and 7 nucleotides long respectively. **BII;** Representation of 7 sites of miRNA-complementary located on positions (89,013,307–89,013,330), (89,013,241–89,013,267), (89,012,867–89,012,893), (89,012,406–89,012,429), (89,012,242–89,012,254), (89,011,703–89,011,726), and (89,011,680–89,011,685) on chromosome 4. Numbers are representative of chromosome location. One of these seed regions is highlighted and magnified below. Also, the seed regions of this miRNA are 6, 6, 6, 7, 6, 6, 6, and 6 nucleotides long respectively (B)·**CI**: the minimum free energy hybridization between miR-548c-3p and 3′UTR of *Tyms*, **CII;** the minimum free energy hybridization between miR-548c-3p and 3′UTR of *Abcg2*.

resistant cell lines derived from HT116 were significantly increased in comparison with the parental HCT116 cell line. The IC50 of HCT116-5FU3, in comparison with the IC50 of parental HCT116, increased 11.27 times (Fig. 3A). The median and range of cell viability in different cell lines are depicted in Fig. 3B. Also, proliferation speed and adherence of the HCT116-5FU3 cell line were reduced in comparison with the parental HCT116. Furthermore, the mortality rate of HCT116-5FU3 was higher than the parental HCT116 cell line. The morphology of HCT116-5FU1 was not different from the parental cell line.

Five generations of SW480 resistant cell lines were generated by consecutive treatment of cells with 5-Fluorouracil. While the IC50 of parental SW480 cell lines was 4.410, the IC50s of SW480–5FU1, SW480–5FU2, SW480–5FU3, SW480–5FU4, and SW480–5FU5 were 12.68, 19.42, 27.00, 34.79, and 41.16 respectively. The IC50s of all resistant cell lines derived from SW480 were significantly higher than the parental cell line. IC50 of SW480–5FU5, in comparison with that in the parental SW480, increased 9.33 times (Fig. 3B). The proliferation speed and adherence of the SW480–5FU5 cell line were reduced in comparison with the parental SW480, SW480–5FU2, SW480–5FU3, and SW480–5FU4. The morphological analysis compared to the parental cell line demonstrated phenotypic changes, such as spindle and elongated shapes of the resistant cell lines in comparison with the parental SW480 cell line (Fig. 4).

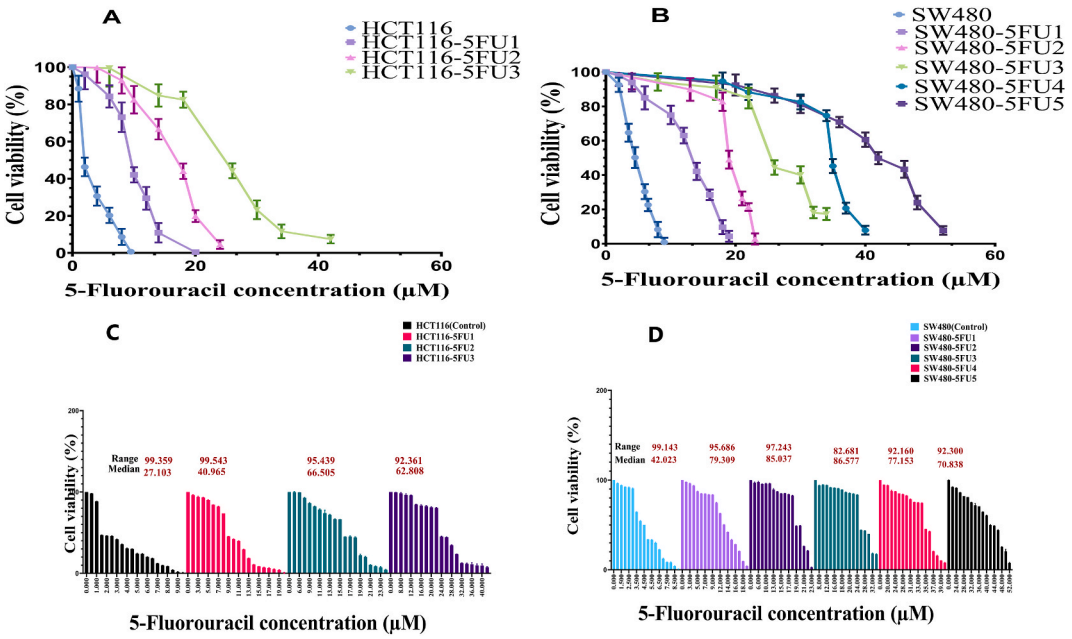


Fig. 3. Cell viability assay of different sub-lines derived from SW480 and HCT116 cell lines after treatment with different concentrations of 5-Fluorouracil (μM). **A**: HCT116 derived sublines in comparison with HCT116. The points are representative of Mean ± SD **B:** SW480-derived sublines in comparison with HCT116. The points are representative of Mean ± SD. **C:** HCT116 derived sublines in comparison with HCT116. Lines are representative of maximum, median, and minimum values. **D:** SW480 derived sublines in comparison with SW480. Lines are representative of maximum, median, and minimum values. Figures are drawn by GraphPad Prism.

3.3. Increased expression of Tyms gene in HCT116-5FU1, HCT116-5FU2, HCT116-5FU3, and SW480–5FU2, SW480–5FU3, SW480–5FU4, and SW480–5FU5 was observed in comparison with the parental cell lines

Fold changes of *Tyms* expression in HCT116-5FU1, HCT116-5FU2, and HCT116-5FU3, were 3.850 ± 0.2, 15.402 ± 0.2, and 19.293 ± 0.3 respectively in comparison with the parental HCT116 (P value < 0.0001). Fold changes of *Tyms* expression in SW480–5FU1, SW480–5FU2, SW480–5FU3, SW480–5FU4, and SW480–5FU5 were 2.770 ± 0.4, 4.07 ± 0.3, 4.155 ± 0.3, 4.112 ± 0.2, and 4.184 ± 0.12 respectively which was significantly different from the parental SW480 cell line (P value < 0.0001) (Fig. 5A).

3.4. Abcg2 expressions in HCT116-5FU1, HCT116-5FU2, HCT116-5FU3, and all SW480-derived resistant cell lines significantly increased in comparison with the parental cell line

Fold change of expression of *Abcg2* in SW480–5FU1, SW480–5FU2, SW480–5FU3, SW480–5FU4, and SW480–5FU5 cell lines in comparison with parental SW80 were 5.52 ± 0.21, 6.38 ± 0.3, 8.08 ± 0.1, 8.25 ± 0.55, and 8.72 ± 0.04 respectively which was significantly different from the parental cell line (P value < 0.0001). The fold changes of expression of *Abcg2* in HCT116-5FU1 and HCT116-5FU2 were 22.47 ± 0.3 and 63.12 ± 0.55 respectively. The fold change of expression of this gene in HCT116-5FU3 increased to 133.90. ±0.6 (p value < 0.0001) (Fig. 5B).

3.5. In all resistant cell lines the expression of miR-548c-3p was decreased

Fold changes of expression of miR-548c-3p in SW480–5FU1, SW480–5FU2, SW480–5FU3, SW480–5FU4, and SW480–5FU5 cell lines in comparison with the parental SW480 cell line were 0.04 ± 0.3, 0.04 ± 0.299, 0.03 ± 0.1, 0.04 ± 0.3, and 0.01 ± 0.5 respectively showing decreased expression of this miRNA in these resistant cell lines.

The fold changes of expression of miR-548c-3p in HCT116-5FU1, HCT116-5FU2, and HCT116-5FU3 were 0.42 ± 0.6, 0.60 ± 0.1, and 0.03 ± 0.4 respectively (Fig. 5C).

3.6. The miR-548c-3p level was inversely correlated with the Tyms and Abcg2 levels in all cell lines derived from SW480 and HCT116

Spearman test was applied to evaluate the association between the level of the predicted miR-548c-3p and the expression level of the *Tyms* gene in all cell lines. The miR-548c-3p level was strongly correlated inversely with *Tyms* expression level in all SW480 and HCT116 derived cell lines (r = - 0.8292, P = 0.0079). According to this data, during the process of resistance induction to 5-Fluorouracil, miR-548c-3p is inversely correlated to the *Tyms* level and vice versa. While the miR-548c-3p level decreased in the resistant cell

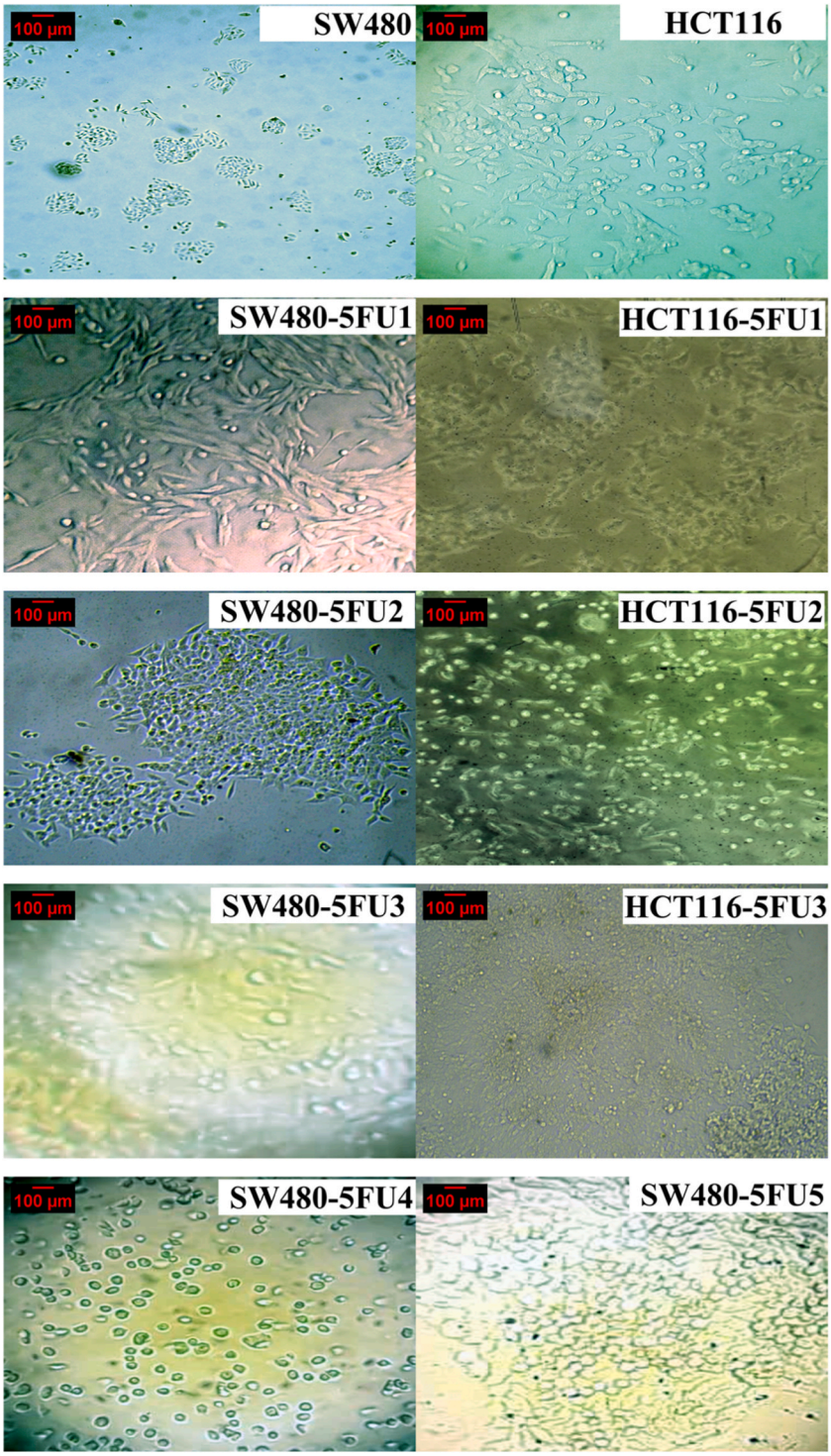


Fig. 4. Cell morphology of parental and resistant sublines of HCT116 and SW480 to 5-FU. The pictures were taken by inverted microscope using a 10× objective lens (× 400; Nikon; Kurobane Nikon Co., Ltd, Otawara, Japan). The scale bar is equal to 100 µm. Pictures were taken after 1–2 passages of cells. These cells are not under treatment by 5-FU and they are taken after resistance induction.

lines, the *Abcg2* level increased in these cell lines. Pearson test was used to investigate the relationship between levels of miR-548c-3p and *Abcg2*. As a result, it was shown that the level of miR-548c-3p was inversely correlated with *Abcg2* level ($r = -0.8091$, $P = 0.0046$) meaning that in all studied cell lines higher levels of miR-548c-3p were correlated to the lower level of *Abcg2* and vice versa. The

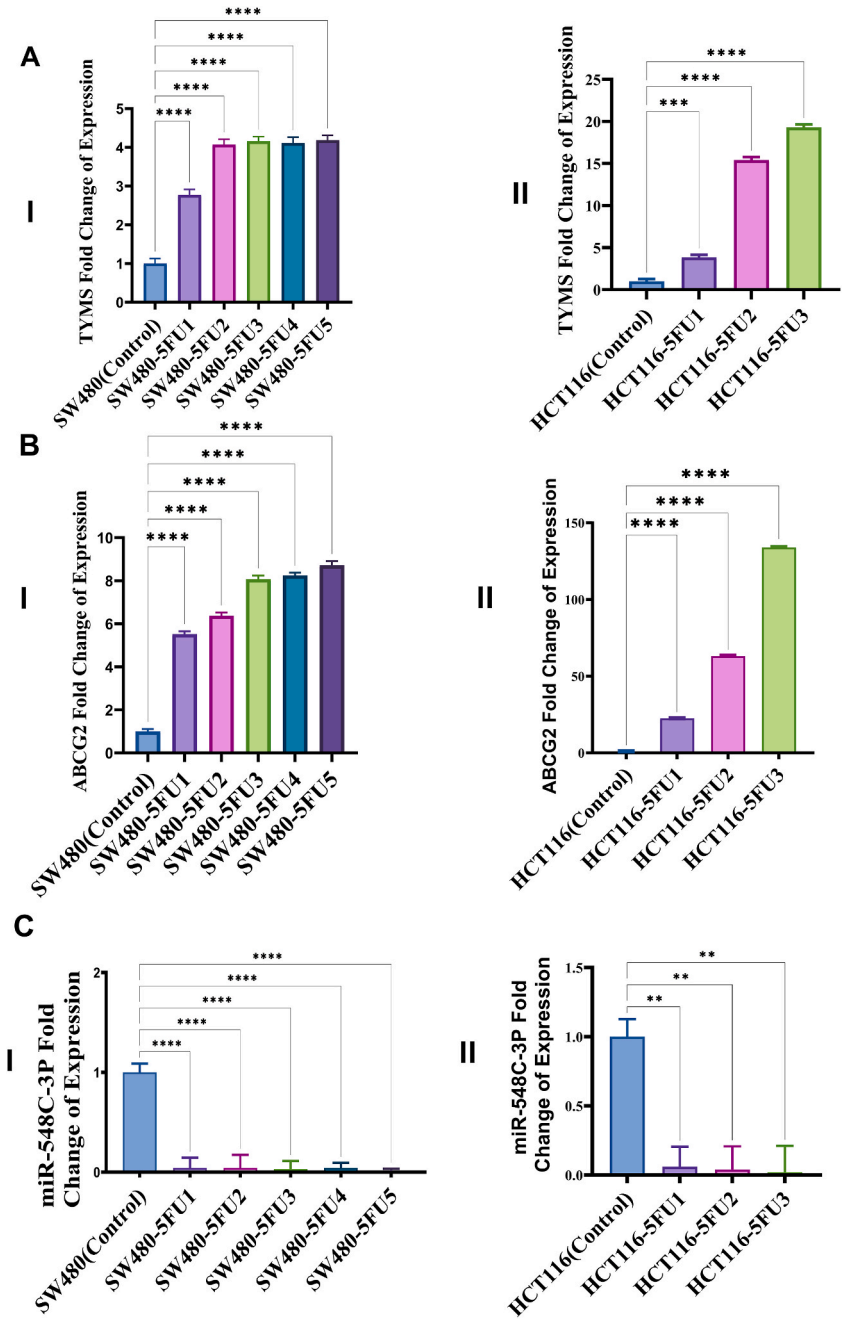


Fig. 5. (A) Real-time PCR-based quantification of *Tyms transcript level* in I: SW480 and II: HCT116 cell lines, (B) Real-time PCR-based quantification of *Abcg2* in I: SW480 and II: HCT116 cell lines, (C) Real-time PCR based quantification of miR-548c-3p in I: SW480 and II: HCT116 cell lines. The data are presented as the mean ± Std. Error of Mean (SEM). * stands for p value < 0.05, ** stands for p value < 0.005, *** stands for value < 0.0005, and **** stands for p value < 0.0001. Statistical analysis was done by Ordinary One-way ANOVA. Figures are drawn by GraphPad Prism.

above-mentioned findings suggest that these two genes are regulated by miR-548c-3p (Fig. 6A).

3.7. IC50 of 5-fluorouracil was positively correlated with the level of Abcg2 and tyms in all HCT116 and SW480-derived cell lines

Pearson correlation analysis was used to investigate whether the increased levels of *Tyms* and *Abcg2* correlated with the expression of *Abcg2* in all SW480 and HCT116-derived cell lines are correlated to the response of cells to 5-Fluorouracil. The results showed that IC50 was correlated positively with the expression of *Tyms* in all SW480 and HCT116-derived cell lines ($r = 0.8754$, $P = 0.0017$). In addition, IC50 was positively correlated with the expression of *Abcg2* in all derived cell lines from SW480 and HCT116 ($r = 0.8389$, P

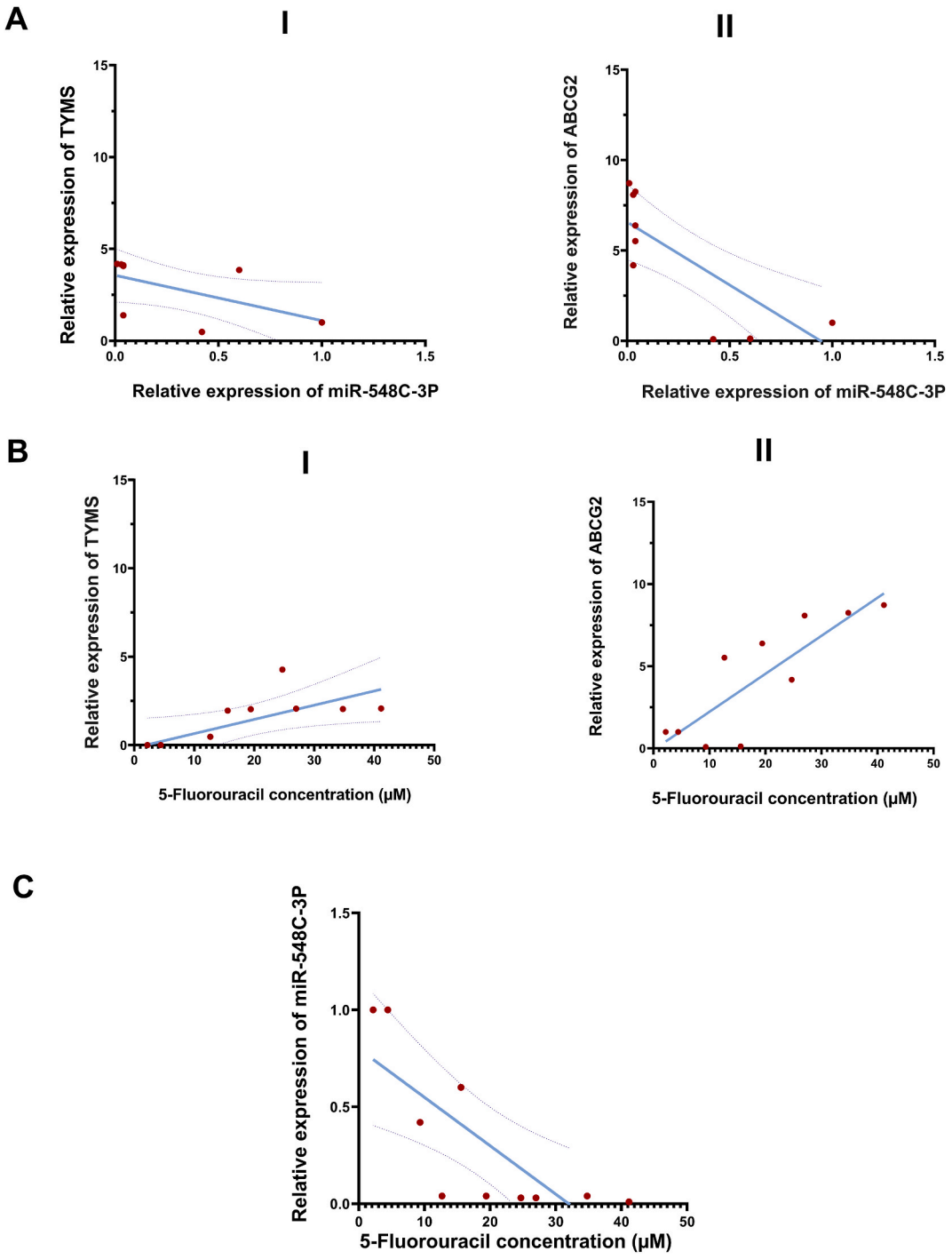


Fig. 6. A (I): Correlation between miR-548c-3p level and *Tyms* transcript level in all cell lines (r = **-** 0.8292, P = 0.0079) **(II):** *Abcg2* transcript level in all cell lines (r = **-** 0.8091, P = 0.0046). **B (I):** Correlation between IC50 to 5-Fluorouracil in all cell lines and *Tyms* transcript level (r = 0.8754, P = 0.0017) **(II):** *Abcg2* transcript level ((r = 0.8389, P = 0.0024). **C (I):** Correlation between the sensitivity of cells to different concentrations of the 5-Fluorouracil and miR-548c-3p level (r = **-** 0.8705, P = 0.0019). Curves were drawn by Graph pad and it shows the correlation between the expression of genes and miRNAs and the 5-Flurouracil IC50. Each dot is representative of the status of different cells according to their sensitivity to 5-FU and expression of the *Tyms* gene/*Abcg2* gene/miR-548c-p. This analysis was done on three replicates of data. Figures are drawn by Graph-Pad Prism.

= 0.0024). It means that during the resistance induction, *Abcg2* and *Tyms* levels increased in accordance with the resistance of cells to 5-Fluorouracil (Fig. 6B).

3.8. IC50 of 5-fluorouracil was inversely correlated with the miR-548c-3p level of in all HCT116 and SW480-derived cell lines

Pearson analysis was performed to investigate the correlation between the sensitivity of cells to different concentrations of 5-Fluorouracil and miR-548c-3p level. The analysis showed that there is a strong inverse correlation between the miR-548c-3p level and IC50 in all HCT116 and SW480-derived cell lines (r = - 0.8705, p = 0.0019). It means that during the resistance induction, the miR-548c-3p le vel decreased consistently with the resistance induction (Fig. 6C).

3.9. MiR-548c-3p increased the sensitivity of SW480 and HCT116 cell lines to 5-FU

To perceive if miR-548c-3p changed the sensitivity of SW480 and HCT116 to 5-FU or not, SW480 and HCT116 were transfected with miR-548c-3p contained plasmid. MTT assay was performed and transfected cells were exposed to various concentrations of 5-FU. It indicated that IC50 of SW480 cells decreased from 4.410 μM to about 2.170 μM in transfected SW480 and, IC50 of HCT116 cells decreased from 2.19 μM to about 1.95 μM in transfected HCT116 (Fig. 7A). Therefore, transfection of cells with miR-548c-3p coincides with the sensitization of cells to 5-FU.

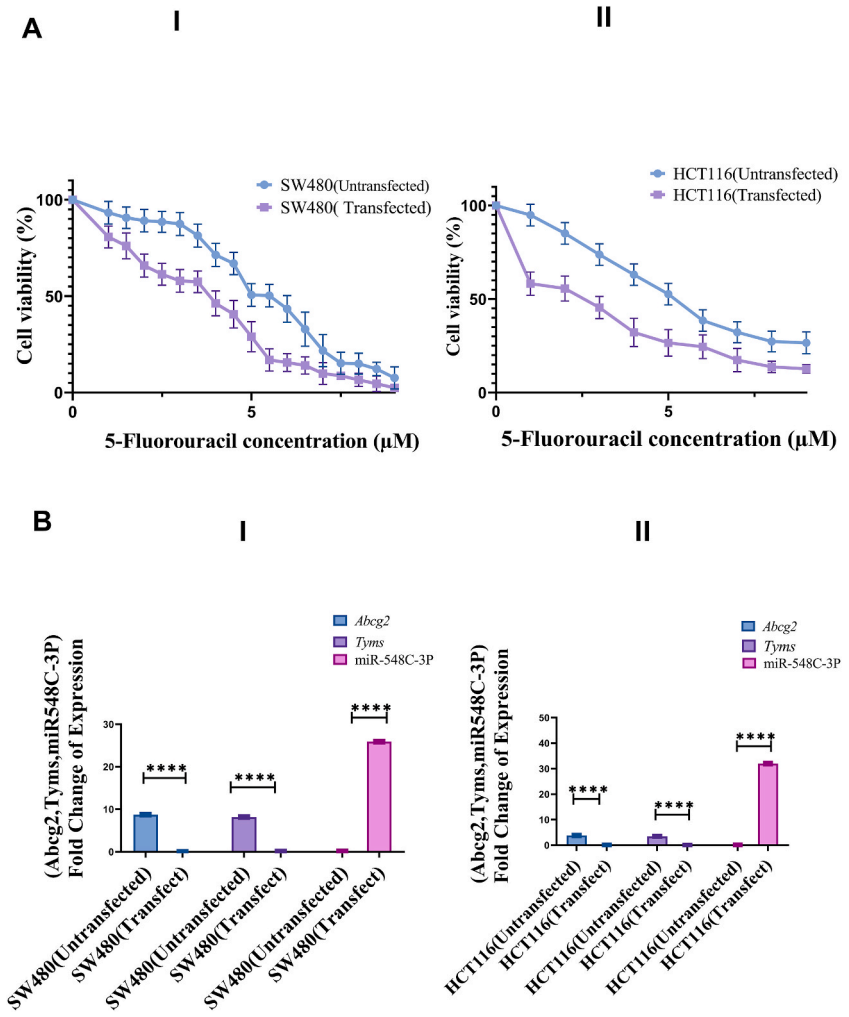


Fig. 7. A: Cell viability assay of transfected (I) SW480 and (II) HCT116 cells and non-transfected with different concentrations of 5-Fluorouracil (μM). B: Real-time PCR-based quantification of *Tyms, Abcg2*, and miR-548c-3p in I: SW480 and II: HCT116 cell lines, the data are presented as the mean ± Std. Error of Mean (SEM). * stand for p value < 0.05, ** stands for p value < 0.005, *** stands for p-value <0.0005, and **** stands for p value < 0.0001. Statistical analysis was done by Ordinary One-way ANOVA.

3.10. Expression of Tyms and Abcg2 was severely decreased following transfection of cells with miR-548c-3p containing plasmid

The association between miR-548c-3p expression and *Tyms* and *Abcg2* transcript level was examined through transfecting of miR-548c-3p to the HCT116 and SW480 cells. It was shown that the level of miR-548c-3p was severely increased after transfection of SW480 andHCT116 with this miRNA, So that, miR-548c-3p increased 32 times in transfected HCT116 cells and 25.90 times in transfected SW480 cells, compared to non-transfected cells. The expression level of *Tyms* gene in transfected HCT116 cells was 0.0000305176 ± 0.31 and in transfected SW480 cells, it was 0.000488281 ± 0.55 similarly, the expression level of *Abcg2* gene in transfected HCT116 cells was 0.0000305176 ± 0.35 and, in transfected SW480 cells it decreased to 0.0000531342 ± 0.2 (Fig. 7B).

4. Discussion

Drug resistance was first observed in the bacteria in response to the antibiotics while a similar phenomenon was also found in the cancerous cells to anti-cancer drugs. Most molecular mechanisms which are involved in the development of drug resistance have been preserved throughout evolution. However, some resistance mechanisms such as absorption, detoxification, and cell receptors may be more important for drug resistance based on the disease and the chemical nature of the drug. Therefore, to check the performance of chemotherapy drugs, in addition to the general mechanisms, the special molecular mechanisms of drug resistance to each chemotherapy drug should be investigated too [7]. 5-FU is the main chemotherapeutic drug used in most chemotherapy regimens, especially for gastrointestinal cancers. Since 1957, 5-Fluorouracil has been widely used in patients diagnosed with CRC. As a result, the long-term use of this drug in diverse populations in terms of race, gender, age, etc., followed by the increase in clinical data, made this drug one of the most useful and interesting research targets. Clinical data about the vast usage of this drug in addition to its efficacy have motivated different researchers to discover the genes and mechanisms in the pharmacokinetics and pharmacodynamics of 5-Fluorouracil.

The response rate to 5-Fluorouracil in the advanced stages of colorectal cancer is 10 %–20 %. In this regard, to seek better treatment strategies, increase the sensitivity of the cells to 5-Fluorouracil, reverse the process of resistance, and identify the genes involved in inducing the resistance, deeper studies are required. This study aimed to investigate the role of one miRNA which is predicted to target *Tyms* and *Abcg2* simultaneously during the resistance induction to sensitize the resistant cells to 5-FU and its application as a sensitivity marker. In this regard, sensitive colorectal cancer cells to 5-Fluorouracil [22]were gradually converted to resistant ones and the expression of *Tyms* and *Abcg2* in addition to the predicted miRNA were studied in these cell lines. The effect of this miRNA on the resistance of cell lines to 5-FU was studied too.

Cancer cells may show either intrinsic or acquired resistance to 5-Fluorouracil. Innate immunity is attributed to the ability of cancer cells to escape the effects of the drug spontaneously in the early stages of drug administration. Acquired resistance has different mechanisms, such as increased drug efflux, epigenetic changes in the genes, drug inactivation, over-activation of DNA damage repair mechanisms, inhibition of cell death, and alteration in the drug targets. It is believed that resistance to 5-Fluorouracil either intrinsic or acquired occurs in nearly half of the patients suffering from metastatic types of colorectal cancer. Studies have shown that thymidylate synthase is one of the key enzymes in 5-Fluorouracil metabolism [31]. The level of this enzyme is the biomarker of sensitivity to 5-Fluorouracil while higher TS is observed in non-responders to 5-Fluorouracil, the lower levels highlight the responders [31,32]. Polymorphisms in the promoter region of this gene like the number of consecutive repetitive sequences determine the level of this enzyme and response to this drug [33–35]. Also, TS has a negative self-regulatory role at the translational level by binding to its mRNA. On the other hand, acquired resistance to 5-Fluorouracil can be acquired from of amplification the TS gene [36]. Abdullah et al. reported that *Tyms* level in the circulating tumor cells would be a predictor biomarker for resistance to 5-Fluorouracil [37]. In this study, in all resistant cell lines, the expression of this gene was increased though, in the three highest resistant cell lines derived from SW480, the expression of *Tyms* was unchanged in comparison with each other. The expression of this gene in the HCT116-5FU3 cell line showed the highest level which is 19 times higher than the parental cell line. In this study, the trend of *Tyms* overexpression accompanies the increased resistance to 5-fluorouracil. Following the transfection of SW480 and HCT116 with miR-548c-3p, IC50 has decreased in comparison to non-transfected cells. Also, the expression of the *Tyms* gene was strongly decreased in cells transfected with miR-548c-3p compared to non-transfected cells. Therefore, miR-548c-3p may serve as a sensitizing agent that sensitizes the resistant colorectal cancer cell lines to 5-FU. The effect of miR-548c-3p on the sensitization of cells to 5-FU may be due to different mechanisms including decreasing the level of *Tyms* though it must be proved by more validation tests. Validating the binding of this miRNA on the 3′UTR of this gene is one of these tests.

Overexpression of the *Abcg2* is considered one of the factors inducing drug resistance in cancers. Cell-based, pre-clinical, and clinical studies have shown overexpression of this gene in several types of cancer, such as colorectal cancer, ovarian cancer, and myeloma. It has been suggested that differences in the expression of this gene can determine the response to different drugs making this gene one of the most important drug transporters involved in drug absorption and elimination [38]. *Abcg2* by the efflux of the drugs out of the cells can prevent intracellular accumulation of the drugs reducing their anti-cancer effects [39]. Overexpressed *Abcg2* was observed in HT29 cells selected with mitoxantrone. Also, in resistant SW1116 cells to hydroxyl-camptothecin, in comparison with the parental SW1116 cells, 200-fold over-expression of *Abcg2* was observed. In vitro studies have shown that *Abcg2* expression was significantly higher in the side population (SP) of colon cancer cells compared to other cells, confirming the higher resistance of SP cells to 5-Fluorouracil and irinotecan [40]. In our study, the expression of this gene in resistant SW480 was increased in comparison with the parental cell line, and the trend of expression of this gene was correlated with IC50 of SW480-derived cell lines. Also, the expression of this gene increased in cells lines derived from HCT116. In addition, the *Abcg2* level after transfecting the cells with miR-548c-3p has decreased sharply. This phenomenon may be due to the effect of miR-548c-3p on decreasing the level of *Abcg2* through its binding to the 3′UTR of this gene. Hence binding of miR-548c-3p to 3′UTR of *Abcg*2 should be validated by further tests.

In this study, we showed that there is a strong positive correlation between *Abcg2* and *Tyms* level and IC50 suggesting the role of these two genes in the process of resistance induction in HCT116 and SW480-derived cell lines.

Most previous studies focused on transcriptional changes, gene amplification, and chromosomal translocation as the main mechanisms of acquired resistance. Nowadays, through increasing knowledge about the role of miRNAs in the expression of genes, more emphasis has been attracted to the role of miRNAs on drug resistance. The role of several miRNAs like miR-519c, miR-328, and miR-520h on the expression of *Abcg2* has been confirmed recently [41]. Also, overexpression of *Abcg2* was found to be correlated with a decreased level of miR-328 in MCF-7/MX100 as a resistant breast cancer cell line [42]. In the human retinoblastoma cell line, it has been demonstrated that decreased expressions of miR-328, miR-519c, and miR-520h were correlated with the increased expression of *Abcg2* [43–46]. In our study, miR-548c-3p downregulation coincides with increased expression of *Abcg2* in resistant cell lines, and this correlation was confirmed in two different colorectal cell lines implying a probable role of this miRNA in downregulating this gene.

Li et al. [46] reported that miR-203 suppresses the expression of *Tyms* leading to an increased inhibitory effect of 5-Fluorouracil. On the other hand, inhibiting the expression of this miRNA increases 5-Fluorouracil drug resistance. Expression of miR-330 reduces cell proliferation and increases the inhibitory effect of 5-Fluorouracil by direct targeting of *Tyms* [47]. Moreover, the expression of miR-218 in HCT116 and HT29 cell lines suppresses the expression of *Tyms* enzyme leading to enhanced apoptosis and sensitivity to 5-Fluorouracil [48]. Boni et al. [49]have shown that the expression of miR-192 and miR-215 which are targeting *Tyms* in colorectal cancer cell lines reduces cell proliferation but does not affect the sensitivity of cells to chemotherapy. In our study, decreased miR-548c-3p accompanies the increased expression of *Tyms* and vice versa suggesting the role of this miRNA in regulating *Tyms* too. This inverse correlation between miR-548c-3p and *Tyms* and *Abcg2* levels implies a possible role of this miRNA in downregulating the level of *Tyms* and *Abcg2* simultaneously and sensitizing colorectal cancer cell lines to 5-Fluorouracil.

It has been previously shown that miR-548c-3p, via inhibition of the HIF1α-mediated VEGF signaling pathway suppresses the progression of papillary thyroid carcinoma [25]. Also, this miRNA blocks migration and invasion of the endometrial and ovarian cancer cells through downregulation of Twist [50]. In another study, it was found that miR-548c-3p with four other miRNAs control the PH-associated genes in the vasculature [51]. Also, miR-548c-3p inhibits lung cancer cell proliferation via the prevention of the Galectin-3-mediated TLR4 signaling pathway.

There is a significant negative correlation between the miR-548c-3p level and IC50, which can indicate the possible role of miR-548c-3p in decreasing drug resistance too. This negative correlation may be related to downregulating the resistance genes such as *Tyms* and *Abcg2*.

We showed that this miRNA may play a role in sensitizing colorectal cancer cells to 5-Fluorouracil through regulating *Tyms* and *Abcg2* gene, which should be validated by further experiments. After validation, this miRNA can be considered as a sensitizing modulator of colorectal cancer cells to 5-Fluorouracil and can be considered as a therapeutic modality. Also, this miRNA can be used as a sensitivity biomarker to 5-Fluorouracil. MiRNAs that regulate the genes involved in drug resistance simultaneously can be promising targets for the modulation of drug sensitivity. This study can be helpful in finding these miRNAs in the future as these miRNAs can be used as drugs without side effects and modulating different aspects of drug resistance in cancerous cells. Studying two important genes involved in drug resistance and finding the miRNA which targets these genes which have been investigated in this study can be beneficial in targeting the resistance genes simultaneously and in a more effective way. In order to validate these results, the binding of mR-548c-3p to the 3′UTR of genes should be studied by different methods. Additionally, the effect of this miRNA on the expression of *Tyms* and *Abcg2* should be studied in other cell lines to have more strong evidence about the role of this miRNA.

5. Data availability statement

Data associated with our study has not been deposited into a publicly available repository. Data will be made available on request.

Ethical consent

No ethical consent was needed for this study.

Funding statement

This study was funded as a Ph.D. student project by Mashhad University of Medical Sciences, Iran (Grant Number: 940863), Biotechnology Development Council (Grant Number: 1240), and Pasteur Institute of Iran, Iran (Grant Number: 95/0110/5503).

Ethics statement

Review and/or approval by an ethics committee was not needed for this study because all study was done experimentally and no animal or human cases were recruited. Informed consent was not required for this study because no patient was recruited in this study.

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Elham Khalili: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Ali Afgar:** Methodology, Formal analysis. **Azam Rajabpour:** Formal analysis. **Seyed Hamid Aghaee-Bakhtiari:** Data curation. **Khadijeh Jamialahmadi:** Supervision, Resources, Funding acquisition, Conceptualization. **Ladan Teimoori-Toolabi:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] M.M. Center, A. Jemal, R.A. Smith, E. Ward, Worldwide variations in colorectal cancer, CA A Cancer J. Clin. 59 (6) (2009) 366–378, https://doi.org/10.3322/caac.20038.

[2] B. Chen, Z. Xia, Y.N. Deng, et al., Emerging microRNA biomarkers for colorectal cancer diagnosis and prognosis, Open Biol 9 (1) (2019), 180212, https://doi.org/10.1098/rsob.180212.

[3] G. Housman, S. Byler, S. Heerboth, et al., Drug resistance in cancer: an overview, Cancers 6 (3) (2014) 1769–1792, https://doi.org/10.3390/cancers6031769.

[4] S. Christensen, B. Van der Roest, N. Besselink, et al., 5-Fluorouracil treatment induces characteristic T>G mutations in human cancer, Nat. Commun. 10 (1) (2019) 4571, https://doi.org/10.1038/s41467-019-12594-8.

[5] D.B. Longley, D.P. Harkin, P.G. Johnston, 5-fluorouracil: mechanisms of action and clinical strategies, Nat. Rev. Cancer 3 (5) (2003) 330–338, https://doi.org/10.1038/nrc1074.

[6] Y. Shen, M. Tong, Q. Liang, et al., Epigenomics alternations and dynamic transcriptional changes in responses to 5-fluorouracil stimulation reveal mechanisms of acquired drug resistance of colorectal cancer cells, Pharmacogenomics J. 18 (1) (2018) 23–28, https://doi.org/10.1038/tpj.2016.91.

[7] W.A. Hammond, A. Swaika, K. Mody, Pharmacologic resistance in colorectal cancer: a review, Ther Adv Med Oncol 8 (1) (2016) 57–84, https://doi.org/10.1177/1758834015614530.

[8] M. Ishikawa, T. Miyauchi, Y. Kashiwagi, Clinical implications of thymidylate synthetase, dihydropyrimidine dehydrogenase and orotate phosphoribosyl transferase activity levels in colorectal carcinoma following radical resection and administration of adjuvant 5-FU chemotherapy, BMC Cancer 8 (2008) 188, https://doi.org/10.1186/1471-2407-8-188.

[9] P. Noordhuis, U. Holwerda, C.L. Van der Wilt, et al., 5-Fluorouracil incorporation into RNA and DNA in relation to thymidylate synthase inhibition of human colorectal cancers, Ann. Oncol. 15 (7) (2004) 1025–1032, https://doi.org/10.1093/annonc/mdh264.

[10] N. Zhang, Y. Yin, S.J. Xu, W.S. Chen, 5-Fluorouracil: mechanisms of resistance and reversal strategies, Molecules 13 (8) (2008) 1551–1569, https://doi.org/10.3390/molecules13081551.

[11] A. Nayak, S. Das, D. Nayak, C. Sethy, S. Narayan, C.N. Kundu, Nanoquinacrine sensitizes 5-FU-resistant cervical cancer stem-like cells by down-regulating Nectin-4 via ADAM-17 mediated NOTCH deregulation, Cell. Oncol. 42 (2) (2019) 157–171, https://doi.org/10.1007/s13402-018-0417-1.

[12] W. Mo, J.T. Zhang, Human ABCG2: structure, function, and its role in multidrug resistance, Int J Biochem Mol Biol 3 (1) (2012) 1–27.

[13] J.T. Zhang, Use of arrays to investigate the contribution of ATP-binding cassette transporters to drug resistance in cancer chemotherapy and prediction of chemosensitivity, Cell Res. 17 (4) (2007) 311–323, https://doi.org/10.1038/cr.2007.15.

[14] D. Westover, F. Li, New trends for overcoming ABCG2/BCRP-mediated resistance to cancer therapies, J. Exp. Clin. Cancer Res. 34 (2015) 159, https://doi.org/10.1186/s13046-015-0275-x.

[15] X. Yu, Z. Li, J. Yu, M.T. Chan, W.K. Wu, MicroRNAs predict and modulate responses to chemotherapy in colorectal cancer, Cell Prolif. 48 (5) (2015) 503–510, https://doi.org/10.1111/cpr.12202.

[16] M. Ghanbarian, A. Afgar, R. Yadegarazari, R. Najafi, L. Teimoori-Toolabi, Through oxaliplatin resistance induction in colorectal cancer cells, increasing ABCB1 level accompanies decreasing level of miR-302c-5p, miR-3664-5p and miR-129-5p, Biomed. Pharmacother. 108 (2018) 1070–1080, https://doi.org/10.1016/j.biopha.2018.09.112.

[17] A. Rajabpour, A. Afgar, H. Mahmoodzadeh, J.E. Radfar, F. Rajaei, L. Teimoori-Toolabi, MiR-608 regulating the expression of ribonucleotide reductase M1 and cytidine deaminase is repressed through induced gemcitabine chemoresistance in pancreatic cancer cells, Cancer Chemother. Pharmacol. 80 (4) (2017) 765–775, https://doi.org/10.1007/s00280-017-3418-2.

[18] G.A. Calin, C.D. Dumitru, M. Shimizu, et al., Frequent deletions and down-regulation of micro- RNA genes miR 15 and miR 16 at 13q14 in chronic lymphocytic leukemia, Proc. Natl. Acad. Sci. U. S. A. 99 (24) (2002) 15524–15529, https://doi.org/10.1073/pnas.242606799.

[19] T. Zheng, J. Wang, X. Chen, L. Liu, Role of microRNA in anticancer drug resistance, Int. J. Cancer 126 (1) (2010) 2–10, https://doi.org/10.1002/ijc.24782.

[20] N. Valeri, P. Gasparini, C. Braconi, et al., MicroRNA-21 induces resistance to 5-fluorouracil by down-regulating human DNA MutS homolog 2 (hMSH2), Proc. Natl. Acad. Sci. U. S. A. 107 (49) (2010) 21098–21103, https://doi.org/10.1073/pnas.1015541107.

[21] H. Chai, M. Liu, R. Tian, X. Li, H. Tang, miR-20a targets BNIP2 and contributes chemotherapeutic resistance in colorectal adenocarcinoma SW480 and SW620 cell lines, Acta Biochim. Biophys. Sin. 43 (3) (2011) 217–225, https://doi.org/10.1093/abbs/gmq125.

[22] S. Cao, L. Lin, X. Xia, H. Wu, MicroRNA-761 promotes the sensitivity of colorectal cancer cells to 5-Fluorouracil through targeting FOXM1, Oncotarget 9 (1) (2018) 321–331, https://doi.org/10.18632/oncotarget.20109.

[23] T. Liang, L. Guo, C. Liu, Genome-wide analysis of mir-548 gene family reveals evolutionary and functional implications, J. Biomed. Biotechnol. 2012 (2012), 679563, https://doi.org/10.1155/2012/679563.

[24] J. Yao, W. Tan, W. Wu, R. Ye, Y. Li, Y. Chen, Effects of miR-432 and miR-548c-3p on the proliferation and invasion of osteosarcoma cells, J BUON 25 (3) (2020) 1562–1568.

[25] Y. Du, J. Zhu, B.F. Chu, Y.P. Yang, S.L. Zhang, MiR-548c-3p suppressed the progression of papillary thyroid carcinoma via inhibition of the HIF1alpha-mediated VEGF signaling pathway, Eur. Rev. Med. Pharmacol. Sci. 23 (15) (2019) 6570–6578, https://doi.org/10.26355/eurrev_201908_18543.

[26] J. Lu, M. Zhang, X. Yang, T. Cui, J. Dai, MicroRNA-548c-3p inhibits T98G glioma cell proliferation and migration by downregulating c-Myb, Oncol. Lett. 13 (5) (2017) 3866–3872, https://doi.org/10.3892/ol.2017.5870.

[27] P.Y. Tan, L.J. Wen, H.N. Li, S.W. Chai, MiR-548c-3p inhibits the proliferation, migration and invasion of human breast cancer cell by targeting E2F3, Cytotechnology 72 (5) (2020) 751–761, https://doi.org/10.1007/s10616-020-00418-3.

[28] A. Afgar, P. Fard-Esfahani, A. Mehrtash, et al., MiR-339 and especially miR-766 reactivate the expression of tumor suppressor genes in colorectal cancer cell lines through DNA methyltransferase 3B gene inhibition, Cancer Biol. Ther. 17 (11) (2016) 1126–1138, https://doi.org/10.1080/15384047.2016.1235657.

[29] L. Teimoori-Toolabi, K. Azadmanesh, S. Zeinali, Selective suicide gene therapy of colon cancer cell lines exploiting fibroblast growth factor 18 promoter, Cancer Biother. Radiopharm. 25 (1) (2010) 105–116, https://doi.org/10.1089/cbr.2009.0643.

[30] L. Teimoori-Toolabi, S. Hashemi, K. Azadmanesh, F. Eghbalpour, F. Safavifar, M.R. Khorramizadeh, Silencing the wild-type and mutant K-ras increases the resistance to 5-flurouracil in HCT-116 as a colorectal cancer cell line, Anti Cancer Drugs 26 (2) (2015) 187–196, https://doi.org/10.1097/CAD.0000000000000175.

[31] P.G. Johnston, H.J. Lenz, C.G. Leichman, et al., Thymidylate synthase gene and protein expression correlate and are associated with response to 5-fluorouracil in human colorectal and gastric tumors, Cancer Res. 55 (7) (1995) 1407–1412.
[32] F. Naghibalhossaini, M. Shefaghat, A. Mansouri, H. Jaberi, M. Tatar, E. Eftekhar, The impact of thymidylate synthase and methylenetetrahydrofolate reductase genotypes on sensitivity to 5-fluorouracil treatment in colorectal cancer cells, Acta Med. Iran. 55 (12) (2017) 751–758.
[33] N. Horie, H. Aiba, K. Oguro, H. Hojo, K. Takeishi, Functional analysis and DNA polymorphism of the tandemly repeated sequences in the 5'-terminal regulatory region of the human gene for thymidylate synthase, Cell Struct. Funct. 20 (3) (1995) 191–197, https://doi.org/10.1247/csf.20.191.
[34] S.T. Pullarkat, J. Stoehlmacher, V. Ghaderi, et al., Thymidylate synthase gene polymorphism determines response and toxicity of 5-FU chemotherapy, Pharmacogenomics J. 1 (1) (2001) 65–70, https://doi.org/10.1038/sj.tpj.6500012.
[35] J. Chen, C. Kyte, W. Chan, J.G. Wetmur, C.S. Fuchs, E. Giovannucci, Polymorphism in the thymidylate synthase promoter enhancer region and risk of colorectal adenomas, Cancer Epidemiol. Biomarkers Prev. 13 (12) (2004) 2247–2250.
[36] A.M. Imam, P.H. Crossley, A.L. Jackman, P.F. Little, Analysis of thymidylate synthase gene amplification and of mRNA levels in the cell cycle, J. Biol. Chem. 262 (15) (1987) 7368–7373.
[37] E.A. Abdallah, M.F. Fanelli, M.E. Buim, et al., Thymidylate synthase expression in circulating tumor cells: a new tool to predict 5-fluorouracil resistance in metastatic colorectal cancer patients, Int. J. Cancer 137 (6) (2015) 1397–1405, https://doi.org/10.1002/ijc.29495.
[38] D.L. Nielsen, J.A. Palshof, N. Brunner, J. Stenvang, B.M. Viuff, Implications of ABCG2 expression on irinotecan treatment of colorectal cancer patients: a review, Int. J. Mol. Sci. (9) (2017) 18, https://doi.org/10.3390/ijms18091926.
[39] L. Chen, J.E. Manautou, T.P. Rasmussen, X.B. Zhong, Development of precision medicine approaches based on inter-individual variability of BCRP/ABCG2, Acta Pharm. Sin. B 9 (4) (2019) 659–674, https://doi.org/10.1016/j.apsb.2019.01.007.
[40] T. Hu, Z. Li, C.Y. Gao, C.H. Cho, Mechanisms of drug resistance in colon cancer and its therapeutic strategies, World J. Gastroenterol. 22 (30) (2016) 6876–6889, https://doi.org/10.3748/wjg.v22.i30.6876.
[41] K.K. To, MicroRNA: a prognostic biomarker and a possible druggable target for circumventing multidrug resistance in cancer chemotherapy, J. Biomed. Sci. 20 (2013) 99, https://doi.org/10.1186/1423-0127-20-99.
[42] Y.Z. Pan, M.E. Morris, A.M. Yu, MicroRNA-328 negatively regulates the expression of breast cancer resistance protein (BCRP/ABCG2) in human cancer cells, Mol. Pharmacol. 75 (6) (2009) 1374–1379, https://doi.org/10.1124/mol.108.054163.
[43] K.K. To, Z. Zhan, T. Litman, S.E. Bates, Regulation of ABCG2 expression at the 3' untranslated region of its mRNA through modulation of transcript stability and protein translation by a putative microRNA in the S1 colon cancer cell line, Mol. Cell Biol. 28 (17) (2008) 5147–5161, https://doi.org/10.1128/MCB.00331-08.
[44] F. Wang, X. Xue, J. Wei, et al., hsa-miR-520h downregulates ABCG2 in pancreatic cancer cells to inhibit migration, invasion, and side populations, Br. J. Cancer 103 (4) (2010) 567–574, https://doi.org/10.1038/sj.bjc.6605724.
[45] K.K. To, R.W. Robey, T. Knutsen, Z. Zhan, T. Ried, S.E. Bates, Escape from hsa-miR-519c enables drug-resistant cells to maintain high expression of ABCG2, Mol. Cancer Therapeut. 8 (10) (2009) 2959–2968, https://doi.org/10.1158/1535-7163.MCT-09-0292.
[46] T. Li, F. Gao, X.P. Zhang, miR-203 enhances chemosensitivity to 5-fluorouracil by targeting thymidylate synthase in colorectal cancer, Oncol. Rep. 33 (2) (2015) 607–614, https://doi.org/10.3892/or.2014.3646.
[47] W. Xu, H. Jiang, F. Zhang, J. Gao, J. Hou, MicroRNA-330 inhibited cell proliferation and enhanced chemosensitivity to 5-fluorouracil in colorectal cancer by directly targeting thymidylate synthase, Oncol. Lett. 13 (5) (2017) 3387–3394, https://doi.org/10.3892/ol.2017.5895.
[48] P.L. Li, X. Zhang, L.L. Wang, et al., MicroRNA-218 is a prognostic indicator in colorectal cancer and enhances 5-fluorouracil-induced apoptosis by targeting BIRC5, Carcinogenesis 36 (12) (2015) 1484–1493, https://doi.org/10.1093/carcin/bgv145.
[49] V. Boni, N. Bitarte, I. Cristobal, et al., miR-192/miR-215 influence 5-fluorouracil resistance through cell cycle-mediated mechanisms complementary to its post-transcriptional thymidilate synthase regulation, Mol. Cancer Therapeut. 9 (8) (2010) 2265–2275, https://doi.org/10.1158/1535-7163.MCT-10-0061.
[50] X. Sun, M. Cui, A. Zhang, et al., MiR-548c impairs migration and invasion of endometrial and ovarian cancer cells via downregulation of Twist, J. Exp. Clin. Cancer Res. 35 (2016) 10, https://doi.org/10.1186/s13046-016-0288-0.
[51] A. Rothman, H. Restrepo, V. Sarukhanov, et al., Assessment of microRNA and gene dysregulation in pulmonary hypertension by endoarterial biopsy, Pulm. Circ. 7 (2) (2017) 455–464, https://doi.org/10.1177/2045893217704206.