miR-192/miR-215 Influence 5-Fluorouracil Resistance through Cell Cycle-Mediated Mechanisms Complementary to Its Post-transcriptional Thymidilate Synthase Regulation

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Abstract

Thymidylate synthase (TYMS) is a target of the most widely used chemotherapeutic agents against gastrointestinal malignancies, the fluoropyrimidine-based therapy. TYMS expression levels have been identified as predictive biomarkers for 5-fluorouracil (FU) response in colorectal cancer, but their clinical utility remains controversial. The complexity of fluoropyrimidine response must require more mechanisms that currently have not been completely elucidated. In this context, microRNAs (miRNA) may play a role in modulating chemosensitivity. By carrying out an in silico analysis coupled to experimental validation, we detected that miR-192 and miR-215 target TYMS expression in colorectal cancer cell lines. However, downregulation of TYMS by these miRNAs does not sensitize colorectal cancer cell lines to FU treatment. The overexpression of miR-192/215 significantly reduces cell proliferation by targeting cell cycle progression. This effect was partially associated with p53 status, because reduction of cell proliferation and cell cycle arrest was associated with p21 and p27 induction. The decrease of S-phase cells by these miRNAs mitigates the effects of S phase–specific drugs and suggests that other mechanisms different from TYMS overexpression are essential to direct FU resistance. Finally, ectopic expression of miR-192/215 might have stronger impact to predict FU response than TYMS inhibition. Prospective studies to elucidate the role of these miRNAs as predictive biomarkers to FU are necessary. Mol Cancer Ther; 9(8); 2265–75. ©2010 AACR.

Introduction

Today, one of the most important aims of oncology research is the identification of new predictive and prognostic biomarkers. Recent works have shown the value of molecular biomarkers to select individuals for various targeted therapeutics, including cetuximab in colon cancer (1). However, equivalent tools to select patients that most likely respond to commonly used chemotherapeutic drugs are lacking.

Nowadays, fluoropyrimidine-based treatments remain a major component of many standard regimens for numerous cancer types and baseline drugs in many experimental regimens with novel agents (2). Despite the introduction of 5-fluorouracil (FU), one of the most important pyrimidine antagonists, in the 1960s and its use in a wide variety of cancer types, at present we still lack useful response predictors to this drug.

FU, an analog of uracil, is an anticancer prodrug that, after administration, is intracellularly converted into 5-fluoro-2-deoxyuridin monophosphate (FdUMP), fluoro-deoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP). The main toxic effects seem to be mediated by thymidylate synthase (TYMS) inhibition through the formation of an extremely stable ternary complex among FdUMP, TYMS, and the cofactor, 5,10-methylene-tetrahydrofolate (CH2FH4; refs. 3, 4). Moreover, the incorporation of FdUTP and FUTP into DNA and RNA significantly contributes to FU cytotoxicity. Several works have previously shown that the mechanism of action of FU may differ depending on the dose and schedule of administration (5, 6). Continuous infusion results in FU metabolism mainly to FdUMP, which then acts as a tightly binding inhibitor of TYMS and inhibits DNA synthesis (4). On the other hand, bolus treatment favors drug metabolism to FUTP and its incorporation into RNA that interferes with normal pre-rRNA processing (7, 8).

TYMS has been historically considered an important molecular biomarker able to predict the response to FU
(9–13). However, the high variability of TYMS expression in colorectal cancer tissues and different methodologies used in previous works did not provide definitive conclusions to recommend the routine use of TYMS expression as a predictive molecular marker to FU treatment (14). One of the most studied mechanisms of TYMS expression variability are functional polymorphisms in the TYMS gene (15). Moreover, it is now well established that regulatory events at the level of gene amplification (16, 17), transcription (18–20), posttranscriptional, and posttranslation (21) are also involved in TYMS expression control (22). One example of TYMS posttranscriptional regulation is the rapid increase in TYMS enzyme activity described in various in vitro (23–25), in vivo, and clinical model systems following short-term exposure to fluoropyrimidines (26, 27).

Based on this evidence, we hypothesized that one mechanism of TYMS posttranscriptional regulation could be mediated by microRNAs (miRNA), a new class of small noncoding RNAs involved in posttranscriptional gene regulation. Many studies have shown the important role of miRNAs as prognostic biomarkers in cancer. Moreover, new findings suggest that miRNAs could be associated with chemosensitivity in tumor cells. Therefore, the potential role of miRNAs in pharmacogenomics is currently an emerging avenue of investigation. Recent works have found that dihydrofolate reductase, a key enzyme in folate metabolism, expression variability are functional polymorphisms in the TYMS gene (15). Moreover, it is now well established that regulatory events at the level of gene amplification (16, 17), transcription (18–20), posttranscriptional, and posttranslation (21) are also involved in TYMS expression control (22). One example of TYMS posttranscriptional regulation is the rapid increase in TYMS enzyme activity described in various in vitro (23–25), in vivo, and clinical model systems following short-term exposure to fluoropyrimidines (26, 27).

Based on this evidence, we hypothesized that one mechanism of TYMS posttranscriptional regulation could be mediated by microRNAs (miRNA), a new class of small noncoding RNAs involved in posttranscriptional gene regulation. Many studies have shown the important role of miRNAs as prognostic biomarkers in cancer. Moreover, new findings suggest that miRNAs could be associated with chemosensitivity in tumor cells. Therefore, the potential role of miRNAs in pharmacogenomics is currently an emerging avenue of investigation. Recent works have found that dihydrofolate reductase, a key enzyme in folate metabolism, is regulated at the translational level by miR-24 (28) and miR-192 (29). The authors showed that the alteration in this mechanism of regulation leads to methotrexate resistance.

We took a systematic approach to identify which miRNAs may target TYMS protein. We showed that miR-192/miR-215 downregulate TYMS expression. However, miR-192/miR-215 are able to modulate cancer sensibility to FU but, given the pleiotropic mechanism of action of miRNAs, mainly by targeting cell cycle progression. Finally, miR-192/miR-215 might be potential candidate biomarkers to predict FU response.

**Materials and Methods**

**Cell culture and reagents**

The human colorectal cancer cell lines RKO, LoVo, DLD1, and SW620 were obtained from the American Type Culture Collection. The DLD1 and SW620 cell lines harbor p53 mutations (mut-p53): a C>T mutation resulting in Ser→Phe at codon 241 and a G>A mutation in codon 273 resulting in an Arg→His substitution, respectively. On the contrary, RKO and LoVo cell lines have a wild-type p53 (wt-p53). In our study p53 mutation status was confirmed by sequencing. All cell lines were maintained in RPMI 1640 (GIBCO-BRL) medium supplemented with 1% penicillin-streptomycin (GIBCO-BRL) and 10% fetal bovine serum (GIBCO-BRL). FU was purchased from Sigma-Aldrich.

**Dual luciferase reporter assays**

For luciferase target assay, the full 3‘ untranslated region (UTR) of the TYMS gene was cloned in the pLR-SV40 vector (Promega). The construct sequence was confirmed by sequencing, HEK293T cells were cotransfected with the reporter constructs (pLR-3’UTR-TYMS), pre-miR-338-5p, pre-miR-192, pre-miR-215, pre-miR-192-mutated, or a negative control (all purchased by Ambion), and pGL3-Promoter vector (Promega), by means of calcium chloride treatment. After 48 hours, cells were harvested and lysed, and ratios between renilla and firefly luciferase activity were estimated using the Dual Luciferase Assay Kit (Promega), according to the manufacturer’s instructions. Data were presented as mean value of renilla/firefly luciferase ratios, obtained from at least three independent experiments.

**Transfections of miRNA and small interfering RNA specific to TYMS**

Pre-miR-192 (50 pmol), pre-miR-215 (50 pmol), and a scrambled control (50 pmol) were transfected into RKO (wt-p53), LoVo (wt-p53), DLD1 (mut-p53), and SW620 (mut-p53) colorectal cancer cells grown in 6-well dishes (plated at 2 × 10³ cells per well 24 hours before transfection). Small interfering RNA (siRNA) specific to TYMS (On-Target plus SMART pool L-004717-00-0005) was purchased from Dharmacon. Transfection was done with Lipofectamine 2000 (Invitrogen). Transfection efficiency (>95%) was confirmed using the Silencer FAM-labeled negative control (Ambion).

**RNA isolation and quantitative reverse transcription-PCR analysis of miRNA**

Total RNA was isolated from cell lines by using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions at 24 or 48 hours after transfection. cDNA synthesis was carried out with the High-Capacity cDNA Synthesis kit (Applied Biosystems) using 10 ng total RNA as template. The miRNA sequence-specific quantitative reverse transcriptase-PCR (qRT-PCR) primers for miR-192, miR-215, and endogenous control RNU6B were purchased from Applied Biosystems. qRT-PCR analysis was carried out using Applied Biosystems 7500 Real-time PCR System. The PCR Master Mix containing TaqMan 2x Universal PCR Master Mix (No Amperase UNG), 10× TaqMan assay, and reverse transcription products in 20 μL volume were processed as follows: 95°C for 10 minutes and then 95°C for 15 seconds, 60°C for 60 seconds for up to 40 cycles. Signal was collected at the end point of every cycle. Gene expression was considered as the ΔΔCT values of the specific miRNAs after normalizing with the internal control RNU6B and relative quantification values were plotted.

**qRT-PCR analysis of mRNA expression**

cDNA was synthesized with the High-Capacity cDNA Synthesis kit (Applied Biosystems) using 2 μg total RNA as the template and random primers. Real-time qRT-PCR was carried out using Applied Biosystems 7500 Real-time PCR System. The PCR Master Mix containing TaqMan 2x Universal PCR Master Mix (No Amperase UNG), 10× TaqMan assay, and reverse transcription products in 20 μL volume were processed as follows: 95°C for 10 minutes and then 95°C for 15 seconds, 60°C for 60 seconds for up to 40 cycles. Signal was collected at the end point of every cycle. Gene expression was considered as the ΔΔCT values of the specific miRNAs after normalizing with the internal control RNU6B and relative quantification values were plotted.
analysis was done on the experimental mRNAs. The PCR primers and probes for TYMS (Hs00926279_g1) and the internal control gene B2M (Hs 99999907_m1) were purchased from Applied Biosystems. qRT-PCR was done on an ABI 7500HT instrument under the following conditions: 50°C for 2 minutes of reverse transcription, 95°C for 10 minutes, 95°C for 15 seconds, 60°C for 1 minute for up to 40 cycles.

Western immunoblot analysis
At 48 hours after transfection with pre-miR-192, pre-miR-215, TYMS-siRNA, or nonspecific control miRNA, the cells were scraped and lysed in radioimmunoprecipitation assay buffer (Sigma). Equal amounts of proteins were resolved by 10% SDS-PAGE gels and transferred to nitrocellulose membranes (Bio-Rad). The membranes were then blocked with 5% nonfat-dry milk in TBS-0.5% Tween-20 at room temperature for 1 hour. The primary antibodies used for the analysis were mouse anti-TYMS (TS106, 1:400, Santa Cruz Biotechnology), mouse anti-p53 (DO-1, 1:1,000, Santa Cruz), mouse anti-p21 (DCS60, 1:2,000, Santa Cruz Biotechnology), rabbit anti-p27 (2552, 1:1,000, Cell Signaling), mouse anti-CDK6 (1:1,000, Santa Cruz Biotechnology), rabbit anti-CDK2 (1:1,000, Santa Cruz Biotechnology), rabbit anti-CCND1 (1:1,000, Santa Cruz Biotechnology), mouse anti-β-tubulin (TU-02, 1:5,000, Calbiochem), and mouse anti-β-actin (ver. 3.1) software was used to analyze the distribution of cells in the different cell cycle stages.

Statistical analysis
All experiments were repeated at least three times. Statistical significance was evaluated by Student's t test (two-tailed) comparison between two groups. The statistical analysis was done with the GraphPad Prism software and with the SPSS 13. Differences were considered statistically significant at P < 0.05.

Results

In silico and experimental identification of TYMS miRNA binding sites
We initially explored the mRNA and protein levels of TYMS in a panel of colon cancer cells. We found that the levels of TYMS miRNA in colon cancer cells did not correlate which TYMS protein expression levels (Supplementary Fig. S1A and B), suggesting that a posttranscriptional regulation of TYMS could be feasible. Thus, we hypothesized that miRNAs could be likely involved in its translational regulation.

To further explore this possibility, we carried out an in silico analysis of those miRNAs that could regulate TYMS expression. Based on the structural analysis of the 3′-UTR of the TYMS gene and using different miRNA predicting target programs (TargetScan, mirBASE, and miRANNA), we identified several miRNAs that potentially interact with the 3′-UTR of the TYMS mRNA. To avoid overprediction, results coming from different databases were intersected and only those miRNAs predicted to target TYMS expression by at least two of the algorithms were considered. Results are shown in Supplementary Table S1.
We first focused on miR-338-5p that was predicted to regulate TYMS by the three algorithms used (Supplementary Table S1). To show a direct interaction between miR-338-5p and TYMS mRNA, we inserted the full-3′-UTR of TYMS mRNA downstream a renilla luciferase reporter cDNA. The HEK293T cells were transfected with this vector together with a pre-miR-338-5p or a control pre-miR. As shown in Fig. 1A, the measurement of renilla activity relative to firefly luciferase did not show a significant repression by miR-338-5p, suggesting that miR-338-5p does not interact with the 3′-UTR of the TYMS mRNA.

Next, we tried miR-192 and miR-215, and we used again the full-3′-UTR of the TYMS mRNA containing the putative binding site for these miRNAs (Supplementary Table S1). In this situation, the measurement of renilla activity relative to firefly luciferase activity showed a significant repression by miR-192 and miR-215 (Fig. 1A).

Figure 1. Identification of miR-192 and miR-215 as regulators of TYMS expression. A, HEK293T cell line was transfected with pre-miR338-5p, pre-miR-192, pre-miR-215, pre-miR-192-mutated, or scrambled control; the renilla reporter vector-3′UTR-TYMS and pGL3-Promoter vector were used as normalization control. The relative renilla/luciferase activity was plotted. Results are represented as mean ± SD for three independent experiments. B, expression analysis of TYMS mRNA by real-time-PCR in RKO, LoVo, DLD1, and SW620 cells transfected with a nonspecific miR control (scrambled), pre-miR-192, pre-miR-215, or TYMS-siRNA. Data are expressed as 2-ΔΔCt values obtained by normalization using β2-microglobulin as endogenous control and cells transfected with a nonspecific miR control as calibrator. C, Western-blot and densitometric analysis of TYMS protein expression in RKO, LoVo, DLD1, and SW620 cells transfected with a nonspecific miR control, pre-miR-192, pre-miR-215, or TYMS-siRNA; β-tubulin was used as protein loading control.
Moreover, to validate the direct regulation of TYMS due to miR-192/215, we showed that the precursor of miR-192 mutated in the “seed region” was unable to bind the predictive 3′-UTR region of TYMS (Fig. 1A). These data indicate that both miR-192 and miR-215 interact with the 3′-UTR of the TYMS mRNA in a specific manner.

**Translational regulation of TYMS by miR-192 and miR-215**

To experimentally confirm that the expression of TYMS was regulated by miR-192/miR-215 in colorectal cancer, the precursors of miR-192 or miR-215 were transfected into RKO, LoVo, DLD1, and SW620 colorectal cancer cells and the expression of the TYMS protein was evaluated by Western immunoblot analysis. To verify the efficiency of transfection, the overexpression of miR-192 and miR-215 was confirmed by qRT-PCR analysis using RNAU6B for normalization (Supplementary Fig. S2). A nonspecific miRNA was used as a negative control and a siRNA for TYMS as positive control. As shown in Fig. 1B, the ectopic expression of miR-192 and miR-215 did not induce an important reduction in TYMS mRNA expression. By contrast, miR-192 and miR-215 clearly decreased TYMS protein expression (Fig. 1C) in all cell lines analyzed.

Moreover, we observed a trend to an inverse correlation between miR-192/miR-215 expression levels and TYMS protein in colorectal cancer cell lines (Supplementary Fig. S1C and D).

These results confirm, in agreement with our initial hypothesis, that a posttranscriptional regulation of TYMS occurs and miR-192 and miR-215 are involved in this regulation.

**miR-192 and miR-215 do not sensitize colorectal cancer cells to FU treatment**

Given that TYMS is the main target for FU treatment, we hypothesized that the increased expression of miR-192 and/or miR-215 might contribute to FU sensitivity by reducing TYMS protein expression. Thus, we used the FU treatment schedule that could be compared with continuous infusion and that has been mainly associated with TYMS inhibition. We tested this hypothesis by an in vitro chemosensitivity assay 48 hours posttransfection with the miRNAs. As shown in Supplementary Fig. S3, overexpression of miR-192 or miR-215 did not change the sensitivity of colorectal cancer cell lines to FU treatment. In contrast, chemosensitivity of RKO, LoVo, and SW620 cells lines treated with the siRNA against TYMS was increased.

Figure 2. Modulation of TYMS expression after FU treatment: Regulation by miR-192/215 transfected cells. A, RKO and LoVo cells were treated with the indicated concentrations of FU. TYMS expression was measured by Western blot and is represented by free TYMS (36 kDa) and ternary complex of TYMS, 5,10-methylenetetrahydrofolate, and FUdUMP (38.5 kDa). β-Tubulin was used as a protein loading control. B, Western immunoblot analysis of free TYMS (36 kDa) and ternary complex of TYMS, 5,10-methylenetetrahydrofolate, and FUdUMP (38.5 kDa) in colorectal cancer cell lines transfected with pre-miR-192, pre-miR-215, TYMS-siRNA, or negative control and treated with 10 umol/L of FU for 24 hours. β-Tubulin was used as a protein loading control.
compared with the corresponding scrambled control cells but not in DLD1 cells. The IC_{50} value for FU was significantly lower in RKO, LoVo, and SW620 transfected with siRNA-TYMS than in the corresponding control cells (1.08 μmol/L versus 0.5 μmol/L for RKO, 0.93 μmol/L versus 0.39 μmol/L for LoVo, and 1.63 μmol/L versus 0.62 μmol/L for SW620).

Although miR-192/215 regulate TYMS at posttranscriptional level, these miRNAs are unable to sensitize colorectal cancer cells to FU. Thus, we investigated which other additional mechanisms mediated by these miRNAs could influence the response to FU. It is known that the active metabolite of FU, FdUMP, forms a ternary complex with TYMS and its reduced-folate cofactor. We explored the effect of miR192/215 on the formation of this ternary complex. We exposed colorectal cancer cell lines to different concentrations of FU for 24 hours. The treatment of the RKO and LoVo cell lines led to the formation of the inhibited ternary complex at 38.5 kDa and continued expression of the free active enzyme at 36 kDa (Fig. 2A). Treatment with 10 μmol/L FU for 24 hours of siRNA-TYMS transfected cell lines completely abolished the formation of the inhibited ternary complex and the free active enzyme (Fig. 2B). We also detected an important reduction in the expression of both TYMS forms (ternary complex and free protein) after FU treatment of miR-192/215 were also able to reduce the inhibited ternary complex of TYMS, although it was not enough to increase FU cytotoxicity.

We finally assumed that miR-192/215 did not increase FU sensitivity due to additional mechanisms mediated by these miRNAs or to the fact that translation of the TYMS protein was not completely inhibited. To explore these hypotheses we used two different approaches. We first increased the time of miR-192 transfection before FU treatment. When we did a transfection with pre-miR-192 for 96 hours, we observed that the overexpression of miR-192 did not sensitize cells to FU but, on the contrary, increased resistance to FU (Fig. 3). In the second approach, we carried out a sequential transfection with pre-miR-192 for 48 hours and subsequent siRNA-TYMS inhibition. In this case, we observed that, although a completely TYMS inhibition was obtained, the overexpression of miR-192 previous to the silencing by siRNA-TYMS was able to reduce the chemosensitivity induced by siRNA-TYMS in RKO and LoVo cell lines. These results show that the ectopic expression of miR-192 decreased FU chemosensitivity, despite complete abrogation of TYMS expression. In this context, we supposed that additional events induced by knockdown effect in proteins targeted by miR-192 are involved in cellular response to FU, independently of TYMS inhibition.

**Overexpression of miR-192 and miR-215 reduces cell proliferation in colorectal cancer cell lines**

While doing the experiments described above, we appreciated that transfection with miR-192 and miR-215 induced important morphologic changes in the colorectal cancer cell lines studied. In response to these miR-NAs, the cells acquired a round shape. In contrast, cells transfected with siRNA-TYMS displayed a similar morphology to control cells (Supplementary Fig. S4).

To assess additional cellular functions of miR-192 and miR-215, we evaluated the effect of both miRNA on cellular proliferation in colorectal cancer cell lines. Our results showed that the overexpression of miR-192 and miR-215 can reduce cellular proliferation in RKO (wt-p53, Fig. 4A),
LoVo (wt-p53, Fig. 4B), and DLD1 (mut-p53, Fig. 4C) with less effect on SW620 (mut-p53, Fig. 4D). These results show the effect of miR-192 and miR-215 on the inhibition of cellular proliferation in a manner partially but not completely p53-dependent pathway.

Effect of miR-192 and miR-215 on cell cycle control

To determine whether the effect of miR-192 and miR-215 on cell proliferation was related to cell cycle control, we analyzed the effect of both miRNAs on cell cycle by flow cytometry.

We pulsed miR-192, miR-215, and siRNA-TYMS transfected cells with the thymidine analogue BrdU to assay the DNA synthesis defects. Our results show that miR-192 and miR-215 significantly increased the number of cells in G0-G1 phase and reduced S-phase cells in LoVo (wt-p53) and RKO (wt-p53) cell lines (Fig. 5A and B). However, in the DLD1 (mut-p53) cell line we did not observe any change in the number of cells in S-phase but we detected an increase of cells in G2-M. In contrast, no effect was observed in the SW-620 cell line (Fig. 5B). These data are in agreement with our previous results with the proliferation assays. On the other hand, we noted that the transfection with the siRNA-TYMS induced a strong arrest of all analyzed cells in the S-phase except for that in DLD-1 cell lines (Fig. 5B). Interestingly, this is the only cell line in which transfection with the siRNA against TYMS did not increase sensitivity to FU (Supplementary Fig. S3). These data strongly suggest that the sensitivity of colorectal cancer cell lines to FU treatment depends on the cell cycle status rather than on TYMS expression. In particular the S-phase of cycle seems to be the major determinant. In this regard, we analyzed BrdU incorporation in the sequential transfection approach (miR-192 and siRNA-TYMS) and we detected an arrest in G1 phase that prevented progression into the S phase and consequently induced a reduction of cells in S phase (Fig. 5C).

Finally, to investigate how these miRNAs affect the cell cycle, we analyzed several key cell cycle–related genes (p53, p21, CCND1, CCNE, CDK4, CDK6, CDK2, and Rb-P). Western blot analysis (Fig. 6A) showed a marked upregulation of p53, p21, and p27 protein expression after ectopic expression of miR-192 and miR-215 in wt-p53 colorectal cancer cell lines (RKO...
and LoVo) but not in mut-p53 DLD1 and SW620 cell lines (Fig. 6B), confirming the link between these miRNAs and p53. As a consequence of p21 and p27 induction, we detected a reduction of hyper-phosphorylation of Rb in wt-p53 cells (RKO and LoVo) but not in mut-p53 cell lines (DLD1 and SW620). Moreover, in LoVo and RKO cells we observed an increased expression of CCND1 and a reduction of CDK6 protein expression without changes in CDK2 or CCNE. On the other hand, in the p53-mutated cell line DLD1 we only detected a significant reduction in the expression of CCNE and an accumulation of CCND1. In contrast, siRNA against TYMS did not change the expression of the proteins involved in cell cycle control.

Discussion

Regulation by miRNAs is obviously not as simple as one miRNA-one mRNA target gene, and knowledge on the gene regulation network by miRNA is just beginning. Typically, more than one miRNA regulates one mRNA, and one miRNA may have several target genes, reflecting a cooperative translational control and target multiplicity. In the present report, we show that miR-192/215 down-regulate TYMS expression posttranscriptionally but due to their pleiotropic effects these miRNAs increase FU resistance in colorectal cancer cell lines. Moreover, we showed that miR-192/miR-215 prevent progression into the S phase and are involved in cell cycle control at multiple levels and subsequently avoid FU cytotoxicity.

It is known that TYMS plays an essential role in catalyzing the reductive methylation of deoxyuridilate (dUMP) to thymidilate (dTMP), which provides the sole intracellular de novo source of dTMP. Due to its biological role, TYMS is critically involved in normal function of the cell cycle and consequently represents an attractive target for anticancer chemotherapy agents such as FU. Although the reason for TYMS expression variability is still unclear, the regulation of the transcriptional and translational activity of this gene likely plays an important role in cell chemosensitivity.

We suppose that miRNAs might be involved in TYMS posttranscriptional regulation. Thus, first by in silico and in vitro expression analysis, we found that miR-192/215 could be potentially implicated in TYMS regulation. We experimentally confirmed that miR-192/miR-215 target TYMS at the 3′-UTR and therefore they might have a key role in the posttranscriptional regulation
of TYMS expression. We reasoned that these miRNAs targeting TYMS could have a relevant effect on FU sensitivity. However, we showed that the reduction of TYMS protein expression was not associated with an increase of FU sensitivity. On the contrary, miR-192 overexpression increased the resistance to FU. Our results highlight the role of miR192/215 as key determinants of chemosensitivity to FU but mainly due to their pleiotropic effect rather than their direct effect upon TYMS expression.

The functional analysis of miR-192/miR-215 revealed that they could induce the accumulation of p53 and its target gene products p21 and p27, and that they suppressed proliferation in a partially but not completely p53-dependent pathway. This observation is supported by our data, showing a decreased growth rate due to miR-192/miR-215 overexpression in the p53-mutated DLD1 cell line. Moreover, we showed that the decrease in cell growth in this mutated-p53 cell line might be explained by cell cycle arrest in the G2-M phase. On the contrary, in wild-type p53 cell lines (RKO and LoVo) transfected with miR-192/215 we detected an important cell cycle arrest in the G1-S phase. In RKO and LoVo cell lines, p21 and p27 induction could inhibit CDK activity and consequently these cells would be unable to phosphorylate pRb, one of the most important cell cycle checkpoints. In DLD1-p53-mutated cell line, we detected a decrease in CCNE protein expression. Therefore, the decrease in cell growth after miR-192/miR-215 overexpression seems to be due to a general regulation of cell cycle genes. In this sense, recent reports showed that miR-192/miR-215 halt cell cycle progression by coordinately targeting transcripts that play critical roles in mediating the G1-S and G2-M checkpoints (30, 31). In addition, to regulate cell cycle-related genes directly, miR-192/miR-215 could also induce arrest by targeting genes that consequently activate the p53-p21 pathway. In cell line models, inactivation of the p21 gene leads to cell cycle arrest failure and apoptosis after DNA-damaging chemotherapy; conversely, overexpression of p21 may lead to the impossibility of the cell to activate S-phase and prevent treatment-induced DNA damage. These data suggest that induction of p21 and/or p27 by miR-192/miR-215 could represent a marker of resistance to chemotherapy. In this sense, recent clinical trials have shown that p21 overexpression (32) and low proliferative index are both tumor characteristics associated with reduced response to FU treatment (33).

Recent works provide new limits to TYMS as a predictive determinant for fluoropyrimidine sensitivity (34), and to date the role of TYMS as a putative predictive and prognostic biomarker in gastrointestinal cancer remains controversial (35–38). In fact, some authors have proposed the proliferation marker Ki-67 as a major determinant of FU clinical response (39). In agreement with these reports, our results highlight the limited role of TYMS expression as predictor marker to FU response and other TYMS-independent events must be involved in FU resistance.

The functional activity of miR-192/215 within colorectal cancer cell lines suggests a potential role of these
miRNAs as tumor suppressors. In agreement with this hypothesis, recent studies have shown that miR-192/miR-215 expression levels were decreased in clinical colon cancer specimens compared with adjacent normal tissues of the same patients (30), indicating that reduced levels of these miRNAs in tumors may be contributing to the malignant phenotype. Moreover, the gene expression profile induced by miR-192/miR-215 is able to control the cell cycle progression (40). It has previously been shown that miR-192 target dihydrofolate reductase expression, a key enzyme of folate metabolism. We have shown that miR-192/215 also regulate TYMS protein expression. We could hypothesize that these targets of miR-192/215, directly involved in DNA synthesis, may affect p53 activity and ultimately alter cell cycle progression.

In conclusion, we provide direct evidence that miR-192 and miR-215 influence sensitivity to FU treatment. Other variables than TYMS expression, such as the cell cycle status, could be considered as major factors that modulate the sensitivity of colorectal cancer cells to FU exposure. Our findings suggest that these miRNAs may be useful FU predictive biomarkers; however, additional studies in the clinical setting are clearly warranted.

Finally, the therapeutic modulation of these miRNAs might be worth exploring in an attempt to improve drug efficacy by overcoming mechanisms of resistance. However, it is mandatory to previously know the complexity of miRNA regulation, because multiple miRNAs may affect multiple genes in a combinatorial fashion, with a fine-tuning depending on the state of the cell. In the future this knowledge should be used to design clinical studies with the aim of delivering more effective treatments to selected patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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