Abstract. Cervical cancer (CC) is one of the most common cancers diagnosed in women worldwide, and it is estimated that ~500,000 new patients are diagnosed with cervical cancer annually and that ~270,000 deaths occur each year. Patients with cervical cancer are treated with different radiotherapy schedules, either alone or with adjuvant chemotherapy. Unfortunately, nearly 50% of all patients with cervical cancer do not respond to standard treatment due to tumor radioresistance. In this scenario, several microRNAs (miRNAs) have been associated with the acquisition of the radioresistance phenotype. The aim of the present study was to evaluate the possible role of miR-125a in the acquisition of radioresistance in cervical cancer. The expression of miR-125a was assessed by means of RT-qPCR in 30 cervical cancer samples from patients receiving standard treatment and 3 induced radioresistant cervical cancer cell lines. In addition, we employed miR-125a mimics and inhibitors to evaluate its function in the induction of radioresistance. We showed that miR-125a was downregulated in patients with cervical cancer who did not respond to standard treatment. Concordantly, radioresistant SiHa, CaSki and HeLa cell lines had low levels of miR-125a with respect to the sensitive cell lines. Finally, we demonstrated that overexpression of miR-125a sensitized cervical cancer cells to radiation therapy through the downregulation of CDKN1A. Our data corroborate previously published studies in which it was demonstrated that miRNAs could play a role in the regulation of the process of radioresistance. Additionally, we showed that overexpression of miR-125a could be used as a radioresistance biomarker in patients with cervical cancer.

Introduction

Cervical cancer (CC) remains one of the most common cancers diagnosed worldwide, and is the third leading cause of cancer-related deaths in women. Approximately 527,600 new patients were diagnosed with CC and ~265,700 deaths occurred in 2012 (1). Standard treatment depends on the clinical stage. However, radiotherapy either alone or with adjuvant chemotherapy remains the standard treatment. Indeed, radiation therapy is utilized for over 60% of patients with CC and is the first treatment choice in ~52% of all patients with cancer (2). Unfortunately, nearly 50% of all patients with CC do not respond to standard treatment due to acquired radioresistance, which is considered the main cause of related deaths associated with treatment failure in patients with CC (3). Thus, radioresistance could be defined as the ability of tumor cells to survive and repair the molecular damage caused by ionizing radiation and its effectors such as free radicals.

MicroRNAs or miRNAs are a group of endogenous, small non-coding RNAs, which are ~21-25 nucleotides in length. miRNAs play a critical role in post-transcriptional gene regulation by degrading or preventing the translation of their target messenger RNA (mRNA). Recently, miRNAs have been called ‘the master regulators’ of gene expression, due to the fact that they have been implicated in a wide variety of cellular processes. To date, few miRNA expression profiles have been related to the radioresistance of patients with CC and CC-derived cell lines; for instance, miR-630, miR-1246, miR-1290, miR-3138, miR-31-3p and miR-3676 were found upregulated, whereas miR-1271, miR-15b*, miR-19b-1*, miR-378*, miR-95, miR-100-5p, miR-200a-5p, miR-320, and miR-342 were found downregulated (4,5). Recently, we
showed that patients with locally advanced CC who do not respond to conventional treatment have a specific miRNA signature, with miR-125 highlighted in that latter signature (6). In the present study, we hypothesized the importance of miR-125a as a potential key regulator for the treatment response of CC patients.

The miR-125 family consists of 3 homologous members: miR-125a; miR-125b-1 and miR-125b-2. These miRNA family members have been linked to several tumors and other chronic degenerative diseases, playing a role as tumor suppressors or oncogenes (7-9).

In the present study, we showed that miR-125a is downregulated in patients with CC who do not respond to standard chemotherapy and radiotherapy treatment. Then, we employed a radioresistance in vitro model employing SiHa, CaSki and HeLa cell lines established by fractionated radiation in order to elucidate the role of miR-125 in the induction of radioresistance. Finally, we demonstrated that overexpression of miR-125a significantly decreased radioresistance through the negative regulation of CDKN1A in CC cells.

Materials and methods

Patient selection and CC samples. We selected 62 tumor samples from the National Cancer Institute of Mexico (INCan) Tumor Bank; 30 fresh-frozen cancer samples were analyzed for miR-125a expression by means of qRT-PCR (15 patients were non-responders and 15, complete responders). Additionally, 32-paraffin-embedded tissues were employed to assess p21 expression by immunohistochemistry (IHC) analysis. All patients included accepted to participate in the study and signed informed consent; the Institutional Ethics and Scientific Board Committees approved the protocol in accordance with The Code of Ethics of the World Medical Association (WMA) (Declaration of Helsinki). All patients were histologically and clinically diagnosed with locally advanced CC [stages IB2-IVA according to the International Federation of Gynecology and Obstetrics (FIGO) classification]. Samples were obtained from patients diagnosed between 2011 and 2014 at the Department of Obstetrics and Gynecology, INCan. All patients had a median of 55 months of clinical follow-up. After sample-taking, the patient samples were categorized into 2 groups depending on their clinical response to standard treatment. Complete response (CR) was defined as the disappearance of all signs of cancer in pathological examination. Standard treatment for patients with locally advanced CC consists of 5 cycles of 40 mg/m² of CDDP (cis-diaminedichloroplatinum II) and a total of 55 Gy of radiotherapy and 30 Gy of internal brachytherapy.

Cell lines. Human CC cell lines SiHa (HTB-35), CaSki (CRL-1550) and HeLa (CCL-2) were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured according to cell-line specifications. CaSki and HeLa cell lines were cultured in RPMI-1640 medium, although the SiHa cell line was cultured in EMEM medium. All cell lines were maintained with 100 U/ml of penicillin and 100 mg/ml of streptomycin, 10% fetal bovine serum, and incubated at 37°C in a 5% CO₂ atmosphere. All cancer cell lines employed in the present study were authenticated by means of the Authentifiler PCR Amplification kit (cat. no. 4479566; Thermo Fisher Scientific, Inc., Waltham, MA, USA) on a 3500 Genetic Analyzer (cat. no. 4440462; Applied Biosystems, Foster City, CA, USA) following the International Cell Line Authentication Committee (ICLAC) guidelines.

Establishment of a radioresistant in vitro model. The human CC cell lines SiHa, CaSki and HeLa were employed to establish a radioresistant cell line model by fractionated radiation. The parental cell lines were grown to 80% confluence, and then the cell lines were trypsinized and divided into 2 subcultures: one for irradiation (RR, radioresistant cells), and the remaining subculture for the non-irradiated condition (RS, radiosensitive cells). When RR culture cells reached 60% confluence, they were irradiated with 2 Gy of X-ray irradiation using an X-ray Linear Accelerator (CL2100C/D; Varian Medical Systems, Palo Alto, CA, USA); immediately after irradiation, the cells were returned to the incubator. Then, after 24 h, the irradiated cell lines were trypsinized and subcultured into new flasks. When they again reached 60% confluence, the irradiation protocol was repeated until the cells reached a total dose of 56 Gy. RS cells were treated under the same conditions as the RR culture, but without irradiation.

Determination of lethal dose 50 of radiation. Lethal dose 50 (LD₅₀) was determined by colony formation assay (CFA). The parental cell lines were incubated at 37°C for 24 h and subsequently were irradiated at different doses ranging from 0-10 Gy. The cells were harvested and counted 24 h after irradiation. Subsequently, 3x10⁵ cells were plated in 6-well culture plates and incubated under standard conditions for 2 weeks. The colonies formed were fixed and stained with glutaraldehyde 6.0% (vol/vol) and crystal violet 0.5% (wt/vol) in water. Finally, colonies consisting of 50 cells or more were counted using an optical microscope and the surviving fraction was determined.

RNA isolation from tumor samples and cell lines. Total RNA from CC tissues and cell lines were extracted and purified with the miRNeasy Mini kit (cat. no. 217004; Qiagen, Inc., Valencia, CA, USA) according to the manufacturer’s instructions. RNA quantification was performed using an Epoch spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

Relative quantification of miR-125 by qRT-PCR. The expression of miR-125 was assessed using the TaqMan MicroRNA assay (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer’s protocol. Briefly, 100 ng of total RNA was subjected to reverse transcription reaction using miRNA-specific RT primers and the TaqMan miRNA reverse transcription kit. The 15-µl reactions were incubated according to the manufacturer’s protocol. Real-time qPCR was performed using TaqMan Universal Master Mix II noUNG in a StepOne qPCR instrument (Applied Biosystems, Carlsbad, CA, USA). Relative expression of miR-125a was calculated utilizing the comparative 2⁻∆∆Ct method. RNU-44 and RNU-6b expression were employed as endogenous control. All of the
Luciferase reporter assays. Reporter plasmids were constructed by ligation of synthetic oligonucleotide duplexes (IDT) containing one of the 3 putative miR-125a target regions in the CKN1A mRNA 3'UTR, including region 1, 5'-CTA GTG GGA GCC CGT CTC AGT GTA-3' and AGC  TTA CAC TGA GAC GGG CTC CCC AGT GCA; region 2, 5'-CTA GTACACAAGGCACCTAGTTCTACCTCGAGGCAA-3'; and region 3, 5'-AGC TTT GCC TGA GGT AGA ACT AGG'-GTG CAC TGG GGA GCC CGT CTC AGT GTA-3'. All putative regions were obtained from microRNA.org and cloned into the pMIR-REPORT plasmid (Ambion Inc., Austin, TX, USA) (10). Each construction was co-transfected with miR-125a mirVana miRNA mimic (Applied Biosystems) and the pMIR-REPORT β-gal control plasmid (Ambion) into HeLa cells. Luciferase activity was analyzed 48 h after transfection utilizing the Dual-Luciferase Reporter Assay System (Applied Biosystems) in a GloMax 96 Microplate Luminometer (Promega, Madison, WI, USA). Luciferase activity was normalized to β-gal activity for each transfected well; each experiment was performed in triplicate.

Western blot analysis. Total protein from cell lysates was extracted using RIPA buffer (sc-24948; Santa Cruz Biotechnology, Inc., Santa Cruz, TX, USA). Then, 50 µg of protein was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Milwaukee, WI, USA) in a Trans-Blot Turbo (Bio-Rad) semi-dry chamber at 25 V and 1 mA for 30 min. After blocking with 5% non-fat milk for 2 h, the membrane was incubated with the specific antibody overnight at 4°C on a rocking platform, washed, and then incubated with the corresponding secondary antibody for 2 h at room temperature. The blot was visualized using the Super Signal West Femto Chemiluminescent substrate (Pierce, Rockford, IL, USA) in a C-Digit scanner (LI-COR)™ employing Image Studio (LI-COR Biosciences, Lincoln, NE, USA) software. The primary antibodies were purchased from Santa Cruz Biotechnology, Inc.: anti-p21 (187) (1:2,000; sc-817). All secondary antibodies were obtained from the Cell Signaling Technology, Inc. (Beverly, MA, USA); anti-mouse (1:5,000; #7076S). β-actin (C4) (1:5,000; sc-47778; Santa Cruz Biotechnology, Inc.) was utilized as an internal control.

Transfection of miRNA mimics and inhibitors. miR-125a mimics and inhibitors were purchased from Ambion and assessed according to the manufacturer's instructions. The pre-miR negative control and scramble oligonucleotide for the miRNA transfection experiments were not homologous to any human miRNA sequences and can be obtained from the Pre-miR miRNA Starter kit (cat. no. Ami1540; Thermo Fisher Scientific, Inc.). Oligonucleotide transfection was performed using the Lipofectamine RNAiMAX transfection reagent (cat. no. 13778150; Thermo Fisher Scientific, Inc.). All experiments were replicated in 6-well plates with a final concentration of 25 pmol of each oligonucleotide, and 7.5 µl of Lipofectamine RNAiMAX was used for each transfection.
Two independent pathologists evaluated the stained sections and the average score for each slide was used for statistical analysis.

**Statistical analysis.** Data are expressed as the mean ± standard error of the mean (SEM) of at least 3 separate experiments performed in triplicate. The differences between groups were analyzed using a double-sided Student's t-test when only 2 groups were present, and the null hypothesis was rejected at 0.01 levels unless otherwise specified.

**Results**

**Radioresistant cell model.** We established a cancer cell line radioresistant model by fractionated irradiation. First, we determined the LD$_{50}$ of radiation for HeLa, CaSki and SiHa cell lines by CFA. The previously mentioned cell lines were irradiated at different doses (0-10 Gy) and survival curves were determined. Therefore, we calculated a ‘parental’ LD$_{50}$ for each cell line. Hence, HeLa cells exhibited a LD$_{50}$ higher than that of the CaSki and SiHa cells (CaSki, 3.0 Gy; HeLa, 3.5 Gy; and SiHa, 2.3 Gy) (Fig. 1A).

After 28 episodes of irradiation, cell lines HeLa-RR, SiHa-RR and CaSki-RR reached a total of 56 Gy, and stable radioresistant cells were obtained from the surviving fraction of parental irradiated cells. Next, LD$_{50}$ for parental cell lines were employed to confirm the radioresistant phenotype in the irradiated cell lines. After parental cell lines were irradiated at LD$_{50}$-calculated doses, as expected, all of the RR subcultures had a higher survival rate than RS cultures. Accordingly, the survival fractions of the HeLa-RR, SiHa-RR and CaSki-RR cells were 58, 62 and 64%, respectively (Fig. 1B). Therefore, we confirmed the establishment of a radioresistant phenotype in the RR subcultures.

**CC samples.** The patients with CC enrolled in the present study had a clinical and pathological diagnosis of locally advanced CC (LACC). All cervical samples were histologically analyzed to confirm a minimum of 80% tumor cells. A summary of the clinical and pathological characteristics of
all patients is presented in Table I. Median age of patients at diagnosis was 52 years (range, 31–68 years). As expected, the most prevalent HPV genotypes identified were HPV-16 (46.8%), HPV-18 (25.8%) and HPV-45 (24.2%).

Relative quantification of miR-125a in CC samples and the radioresistant cell model. We recently published an expression profile of miRNA associated with clinical response in patients with CC under standard treatment of chemotherapy and radiotherapy; one of these identified miRNA was miR-125a (6). Based on this evidence, we aimed to elucidate the functional role of miR-125a in the radioresistance phenotype in CC. Hence, we assessed the expression level of miR-125a in 30 dichotomized CC samples (NR=15 and CR=15). Relative expression of miR-125a was significantly lower in the 15 NR samples with regard to the CR samples (P≤0.0001) (Fig. 2A).

We quantified the expression level of miR-125a in HeLa-RR, SiHa-RR and CaSki-RR subcultures (Fig. 2B). miR-125a was underexpressed in all RR subcultures with respect to RS cell lines.

It is noteworthy that miR-125a was found 5-fold underexpressed in the CaSki RR cell line with respect to the CaSki RS cell line (P=0.0001), whereas in HeLa-RR and SiHa-RR cell lines, miR-125a was found up to 2.5-fold underexpressed (P=0.001 and P=0.027, respectively). We conducted the following experiments on the HeLa cell line in order to achieve a better performance of transfection assays, such as that reported by Asgharian et al (11).

Molecular targets of miR-125a. After confirming that miR-125a was underexpressed in resistant CC samples and radioresistant (RR) CC cell lines, we conducted an exhaustive search on the most citable bioinformatics algorithms for predicting miR-125a targeted mRNA (microRNA.org, TargetScan and miRDB). Five hypothetical target genes of miR-125 were obtained by means of this method (CDKN1A, SP1, E2F7, CDKN1A, and RAF1).

Figure 2. Relative expression of miR-125a, CDKN1A and RAF1. (A) Relative expression of miR-125a was analyzed in 30 patients with CC [complete response (CR)=15, and no response (NR)=15]. Each dot represents the relative expression level for a single patient by triplicate. Relative expression was normalized with RNA-U6- and RNU-44-level expression. (B) Relative expression of miR-125a was evaluated in CaSki, HeLa and SiHa cell lines. miR-125 was downregulated in CaSki (P=0.0001), HeLa (P=0.001) and SiHa (P=0.027) cell lines. (C) CDKN1A, a molecular target of miR-125a, was upregulated in the HeLa-RR cell line, with respect to the HeLa-RS cell line (P=0.01). (D) RAF1, a molecular target of miR-125a, was upregulated in the HeLa-RR cell line with respect to the HeLa-RS cell line (P=0.048).

Figure 3. Relative expression of CDKN1A in HeLa-RR and HeLa-RS cell lines. (A) Relative expression of CDKN1A was evaluated in a radioresistant (RR) HeLa cell line with regard to radiosensitive (RS) parental cells. The CDKN1A gene was upregulated in the HeLa-RR cell line (P=0.035). Additionally, the CDKN1A gene was deregulated when the HeLa-RS cell line was irradiated (P=0.002).
AKT1 and RAF1), which were tested by qRT-PCR to suggest a possible regulation by miR-125a. We found an overexpression of CDKN1A and RAF1 (Fig. 2C and D) transcripts in the HeLa cell line (P=0.01 and P=0.048), respectively, whereas, differences in SP1, E2F7 and AKT1 mRNA were not statistically significant (data not shown). Additionally, we demonstrated that CDKN1A is overexpressed after LD50 irradiation doses, in both RS and RR cells (Fig. 3), suggesting a possible role of CDKN1A in the radioresistant phenotype.

Western blot analysis of p21. The protein levels of p21 (CDKN1A) were assessed in the HeLa-RS and HeLa-RR cells. Our results confirmed that p21 was overexpressed by up to 3-fold in the HeLa-RR cells with regard to the HeLa RS cells (P=0.019). In addition, the radiation increased p21 protein levels 2-fold in the RS cells (P=0.001). It was encouraging to note that in all cases where the cells were irradiated, p21 was overexpressed (Fig. 4A and B). RR cells had higher levels of CDKN1A, probably due to a downregulation in the expression...
of miR-125a. In order to test this hypothesis, we restored the expression of miR-125a by means of a mimic sequence transfected into RR cells. As observed in Fig. 4C and D, restoration of miR-125a by a mimic sequence decreased the protein levels of p21, suggesting a direct role for CDKN1A regulation exerted by miR-125a.

**miR-125a regulates the expression of CDKN1A.** To validate whether CDKN1A mRNA is a molecular target of miR-125a, we performed a luciferase reporter assay. Three putative binding sites for miR-125a in the CDKN1A 3'UTR were cloned into the pMIR-REPORT plasmid. The binding regions between miR-125a and CDKN1A mRNA comprise the following: 1, 545-568; 2, 769-805, and 3, 1266-1287 nucleotides into the 3'UTR of the p21 transcript (Fig. 5A). After normalization with the β-gal control, luciferase activity was suppressed in all 3 binding sites cloned. These results demonstrated that the 3 cloned miR-125a-binding sequences are usable for inhibition of p21 transcript expression by miR-125a (Fig. 5B).

**P-21 expression in CC tissues.** In order to confirm that p21 could be a RR marker in CC tissues, we assessed protein expression by means of IHC in 32 paraffin-embedded tissues (in 15 CR and in 17 NR tissue samples). We observed a slight difference in the p21 protein expression level between both sample groups without a statistically significant significance (Fig. 5C). We hypothesized that the number of samples analyzed by IHC did not allow corroborating a higher level of the p21 protein in patients diagnosed as non-responders to standard chemotherapy and radiotherapy treatment.

**miR-125a sensitizes CC cell lines to radiation therapy.** To elucidate whether overexpression of miR-125a sensitizes CC cells to irradiation treatment, we transfected miR-125a mimics and inhibitors into HeLa cells and determined the sensitivity of transfected cells after the irradiation dose. First, we corroborated the level of expression of p21 in the transfected cells (Fig. 4C). Then, after 24 h, we performed a post-irradiation CFA and cell survival fractions were calculated after 2 weeks of incubation. Notably, we found a significant decrease in survival fractions in the miR-125 mimic-transfected cells (Fig. 6A) compared to their control cell lines (negative and scramble oligonucleotide conditions) (P=0.005). When cells were transfected with the miR-125 inhibitor we found a higher number of colonies (Fig. 6B; P=0.001) in respect to their respective negative and scramble control cells. These findings strongly suggest that miR-125a is a regulator of acquired radioresistance in CC.
Discussion

Recently, several research groups have reported that the radioresistance phenotype in cell lines and tumor samples could be explained by the expression profile of miRNAs. Notably, these studies have listed some miRNAs as possible regulators of the radioresistant phenotype (12-14). However, only a few of these have been assessed by functional assays to demonstrate a possible regulatory role. In the present study, we demonstrated that miR-125a regulates the expression of p21 and that this event is related to the sensitization of CC cells to radiation therapy. The acquisition of radioresistance is a complex process in which a wide number of genes and several signaling pathways are involved (15-17). A single miRNA regulates several hundreds of genes that could be implicated in the radioresistance phenotype. Due to this, miRNAs possess evident advantages for their consideration as radioresistance modulators based on their ability to control multiple targets in specific molecular pathways.

Here we showed that miR-125a is downregulated in patients with cervical cancer diagnosed as non-responders (NRs) to conventional therapy (Fig. 2A). Moreover, these results were validated in vitro employing radiosensitive (RR) and radiosensitive (RS) HeLa, SiHa and CaSki cervical cancer cell lines (Fig. 2B). This evidence indicates a correlation between the expression level of miR-125a and the radioresistant phenotype in cervical cancer.

Recently, Moskwa et al identified a subset of miRNAs associated with radioresistance in glioblastoma cell lines. Among the identified miRNAs there were the following: miR-1; miR-150; miR-425; and notably, miR-125a (18). In addition, these authors demonstrated, by functional assays, that miR-125a promotes radioresistance in LN229 and U251 cell lines. In this same research line, Shiiba et al demonstrated the participation of miR-150; miR-425; and notably, miR-125a (18). In addition, among the identified miRNAs there were the following: miR-1; miR-150; and notably, miR-125a (18). In addition, these authors demonstrated, by functional assays, that miR-125a regulates the expression of p21 and that this severely affects its cellular ability to respond to radiation therapy (Fig. 6A and B). These findings and those previously published by other authors support the idea that miRNAs could function as central regulators of radioresistance acquisition by cancer cells. Our findings determined a specific role for miRNA-125a in the radioresistance of cervical cancer, and it may be considered for future therapeutic strategies for this neoplasia.

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References


