RESEARCH ARTICLE

Screening for MiRNAs Related to Laryngeal Squamous Carcinoma Stem Cell Radiation

Chang-Xin Huang[1]*, Ying Zhu[2], Guang-Liang Duan[1], Ji-Fen Yao[1], Zhao-Yang Li[1], Da Li[3], Qing-Qing Wang[3]

Abstract

Objective: To use microarray chip technology for screening of stem cell radiation related miRNAs in laryngeal squamous cell carcinoma; study and explore the relationship of miRNAs with radiosensitivity of laryngeal squamous cells. Method: After conventional culture and amplification of the laryngeal squamous carcinoma cell line Hep-2, CD 133+ cells were screened out with combination of isolated culture of stem cell microspheres and FACS for preparation of laryngeal cancer stem cells. After radiation treatment, miRNAs of laryngeal squamous carcinoma stem cells before and after radiation were enriched and purified. After microarray hybridization with mammalian miRNA and scanning of fluorescence signal, the miRNAs of laryngeal squamous carcinoma stem cells before and after radiation was subject to differential screening and clustering analysis. Real-time quantitative RT-PCR was used to verify part of the differentially expressed miRNAs. Results: 70 miRNAs related to laryngeal cancer stem cell radiation with 2-fold difference in expression were screened out, in which 62 were down-regulated and 8 were up-regulated. Fluorescent quantitative RT-PCR results were consistent with miRNAs chip results. Conclusion: Some miRNAs may be involved in self-regulation with laryngeal squamous carcinoma stem cell radiation.

Keywords: Laryngeal carcinoma stem cells - miRNA - radiation related - differential expression

Asian Pac J Cancer Prev, **14 (8)**, 4533-4537

Introduction

Cancer stem cells are considered to be the source of tumor development and radiation resistance (Hambardzumyan et al., 2006; Blazek et al., 2007; Kang et al., 2008; Ghotra et al., 2009). In recent years, people have successfully separated and identified dozens of cancer stem cells; on the basis of isolated and identified tumor stem cells, some research has gone deep into the level of gene regulation in cancer stem cells. MicroRNA (miRNA) is a newly discovered short non-coding sequencing single-stranded RNA molecule in recent years. It is currently the most important class of gene regulatory molecules in known tumor cells. Specific miRNAs play a unique and important regulation role in characteristics of cancer stem cells (Schetter et al., 2008; Avissar et al., 2009; Hime and Somers, 2009; Mueller et al., 2009; Valeri et al., 2009). including the regulation of genes with biological characteristics such as radiation tolerance gene. It can be inferred that radiation-specific miRNAs play an important role in the regulation of radiation resistance related genes of cancer stem cells.

Radiotherapy is one of clinically important treatments of laryngeal cancer. However, the radiation resistance of laryngeal cancer cells is still a difficult problem which seriously affects radiotherapy. Currently, studies of miRNA regulation on the radiotherapy resistance level of laryngeal cancer stem cells have not been reported both home and abroad. Because of the fast, parallel, high-throughput detection characteristics, we selected oligonucleotide microarrays of miRNA expression profiles for comparative analysis of miRNA expression level in laryngeal cancer stem cells before and after radiation, differential miRNA screening and clustering analysis; part of the differentially expressed miRNAs was verified with quantitative real-time RT-PCR method. This laid the foundation for further study of the relationship of miRNA with laryngeal cancer radiation related biological characteristics and provided basic information on the development of laryngeal cancer stem cell radiosensitizer based on miRNA regulation and targeting.

Materials and Methods

Materials

Hep-2 laryngeal squamous carcinoma cell line was purchased from Shanghai Institute of Cell Institute. CDl33 + monoclonal antibody was purchased from Chemicon

*[1]Department of Oncology, Affiliated Hospital with Hangzhou Normal University School of Medicine, [2]Second Clinical Medical College of Zhejiang Chinese Medicine University, [3]Institute of Immunology, Zhejiang University, Hangzhou, China *For correspondence: hcx588@hotmail.com, hcx588@tom.com*

company; goat anti-rabbit IgG/FITC was purchased from Toxin Tech.

Construction of laryngeal stem cells

Conventional isolation culture of stem cell microspheres: Hep-2 laryngeal squamous carcinoma cell line was cultured in medium containing serum l640; serum-free medium (SFM) was prepared freshly, that is EGF, bFGF, and glucose were added in serum-free DMEM/F12 medium. Laryngeal cancer cells in growth stage were subject to trypsin digestion and SFM resuspention into 10^3/ml single cell suspension, followed by routine culture in glass culture flasks. Culture bottles were placed upright in an incubator and shaken a few times a day. The microsphere formation was observed. The medium was changed every 2 days at half the amount and passaged every 6 days. Microspheres were collected during passage, and were subject to accutase digestion and pipetting into single cells. After washing in SFM they were subcultured for amplification and enrichment of laryngeal stem cells. All growing cells ball were collected and made into single cell PBS suspension. Fluorescent-labeled monoclonal antibody was added and incubated. CDl33 + cells were screened out as laryngeal cancer stem cells using FACS.

Radiation treatment of laryngeal cancer stem cells

Radiation processing method for tumor cells commonly used in similar studies was applied: Laryngeal cancer stem cells SFM suspension with cells adjusted to 1×10^6/ml, was inoculated in 6-well culture plate. After short-term culture, the cultured cells were irradiated with linear accelerator. The condition was vertical 6MV-X-ray irradiation and 2 Gy/min dose rate. During irradiation, the bottom of the culture plate was added 1.5 cm tissue equivalent filler and the source skin distance (SSDs) was 100cm. The irradiation was carried out twice with 5Gy/ times and the irradiation interval was 72 h.

Screening of different miRNA

miRNA The Ambion miRNA isolation kit was used for enrichment and purification of miRNA: Conventionally extracted total RNA and 5 times volume of lysis/binding buffer solution was mixed well and was added 1/10 volume of miRNA homogenate additives. The mixture was incubated on ice and was added 1/3 volume of anhydrous ethanol. It was passed through filter tube after mixing. The filtrate was added 2/3 volume of anhydrous ethanol and preheated to 95 °C followed by second filtration. Labeled reactant was prepared with bovine intestinal alkaline phosphatase, and miRNA was fluorescently labeled with T4 ligase and purified with column. Fluorescent miRNA sample was added 10 × GE blockers and 2 × HiRPM hybridization buffer, and the miRNA chip (Denmark Exiqon Company) hybridization was carried out with hybrid instrument and its rotary oven. Upon finish each chip was washed in GE buffer and scanned with Agilent microarray scanner to obtain the hybridization signal.

Chip signal reading and data processing

LuxScan3.0 image analysis software (CapitalBio, China) was used for mircoarray image analysis. After overall normalization of data and logarithmic transformation, Significance Analysis of Microarrays (SAM, version 2.1) was used to select differentially expressed genes. The screening requirement was FDR was controlled in less than 5%, and Fold change was not less than 2.0. Finally, Cluster3.0 (Stanford University) was adopted for cluster analysis.

Fluorescence quantitative PCR

RNA-tailing and primer extension RT-PCR was applied for quantitative real-time detection of miRNAs in stem cells: First establishing 20 μl tailing reaction system: 1 μg total RNA, poly A polymerase (PAP) 40 U, 1 mmol/L ATP at 37 °C water bath for 1h. Then, an equal volume of phenol-chloroform (1:1) extraction followed by centrifugation at 12000 r/min for 10 min to afford the supernatant. 1/l0 Volume of 3 mol/L NaAc and 2 volumes of anhydrous ethanol was added to precipitate RNA. RNA after tailing was dissolved in 10μl RNA-free enzyme water. Reverse transcription reaction was carried out with transcription kit, and the reverse transcription specific primer was the universal primer sequence binding to the polyA end in RACE kit. According miRNA sequences to be detected, PCR upstream primer was designed and synthesized. The common downstream primer: 5'-GCTGTCAACGATACGCTACGTAACG-3'; 5SRNA was selected as internal reference; upstream primer: 5'-CCATACCACCCTGAACGC 3'; downstream primer: 5'-AGCCTACAGCACCCGGTAT-3'. Real-time PCR amplification adopted crystalline core EvaGreen fluorescence quantitative PCR Universal kit of CapitalBio Company. In PCR amplification, primer concentration was 150 nmol/L. PCR cycling condition was: 94°C predenaturation for 5 min, 94 °C denaturation for 30 's, 58°C annealing for 30S, 72 °C extension for 45S, 74 °C plate reading, a total of 40 cycles, three duplicate for each miRNA. The specificity of the PCR reaction was confirmed with product melting curve, and the relative content of mRNA was calculated according to the formula: Relative fold chang = $2^{-\Delta(\Delta Ct)}$. where in ΔCt = Ct(target)-Ct(5sRNA); $\Delta(\Delta Ct) = \Delta Ct(test) - \Delta Ct(control)$.

Results

The successful separation construction of laryngeal squamous cancer stem cells

Conventional isolated culture of stem cell microspheres: Laryngeal cancer cells in growth stage were subject to trypsin digestion and SFM resuspention into 10^3/ml single cell suspension followed by routine culture in glass culture flasks. Culture bottles were placed upright in an incubator, and were shaken a few times a day. The microsphere formation was observed. The medium was changed every 2 days at half the amount and passaged every 6 days. Microspheres were collected during passage (Figure 1). and were subject to accutase digestion and pipetting into single cells. After washing in the SFM they were subcultured for amplification and enrichment of laryngeal stem cells. All growing cells ball were collected and made into single cell PBS suspension. Fluorescent-

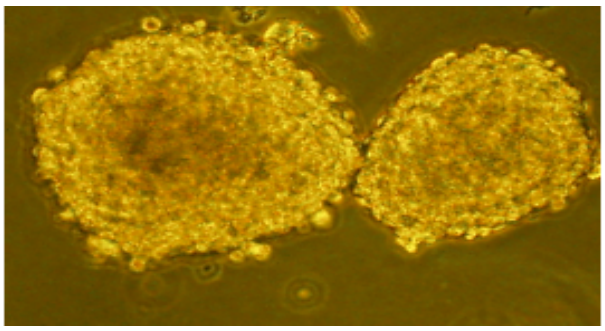

Figure 1. Spheroids of Enriched Laryngeal Stem Cells (× 200 times). Laryngeal cancer cells in growth stage were subject to trypsin digestion and SFM resuspention into 103/ml single cell suspension followed by routine culture in glass culture flasks. Culture bottles were placed upright in an incubator, and were shaken a few times a day. The microsphere formation was observed

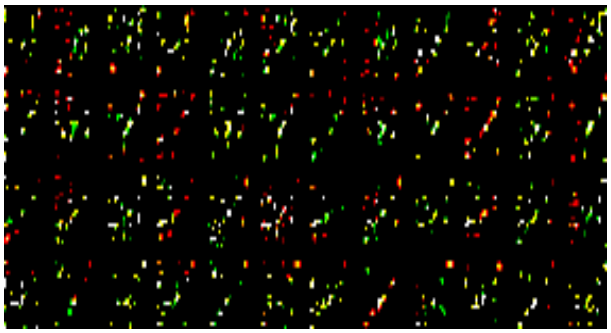

Figure 2. Fluorescently Labeled miRNA Microarray Hybridization. Trizol method was applied to isolate total RNA before and after laryngeal cancer stem cells irradiation. After enrichment miRNA was detected and showed A260/A280 values were in the range of 1.8 to 2.1, in line with the requirements of the miRNA microarray. Such sample can be used for miRNA microarray experiments. After hybridization of labeled and purified miRNA with microarray chip, it was scanned to afford images at 543 nm channel

labeled monoclonal antibody was added and incubated. CDl33 + cells were screened out with FACS as laryngeal cancer stem cells. 2 mL of stem cell suspension liquid was obtained with 1 × 10^7/ml concentration of cells.

Figure 1 Spheroids of enriched laryngeal stem cells (× 200 times): Laryngeal cancer cells in growth stage were subject to trypsin digestion and SFM resuspention into 10^3/ml single cell suspension followed by routine culture in glass culture flasks. Culture bottles were placed upright in an incubator, and were shaken a few times a day. The microsphere formation was observed.

Hybridization results

Trizol method was applied to isolate total RNA before and after laryngeal cancer stem cells irradiation. After enrichment miRNA was detected and showed A260/A280 values were in the range of 1.8 to 2.1, in line with the requirements of the miRNA microarray. Such sample can be used for miRNA microarray experiments. After hybridization of labeled and purified miRNA with microarray chip, it was scanned to afford images at 543 nm channel. Positive control signals such as HEX, external standard, internal standard were normal and the negative control test was negative. The microarray chip

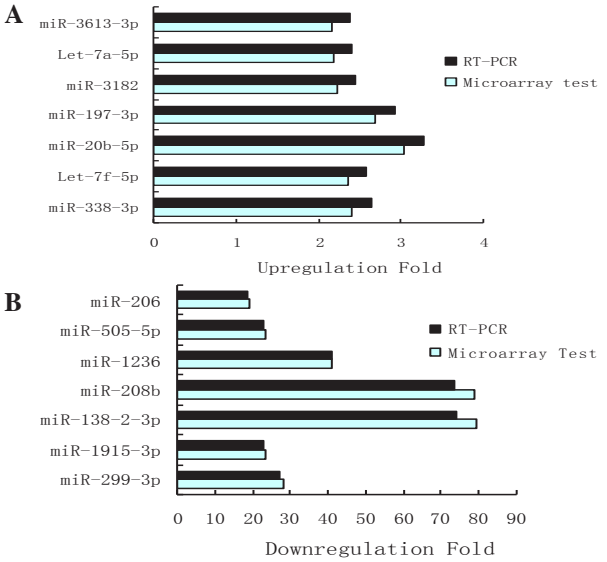

Figure 3. Some miRNA Expression Detected by qRT-PCR and the Chip. Eva Green quantitative real-time PCR amplification was applied to detect the expression of some miRNA that showed differential expression before and after irradiation in chips. The chip results and fluorescence quantitative RT-PCR results were compared and the results showed consistent expression of differential miRNA detected from chip and quantitative RT-PCR

had good repeatability of duplicate points and uniform signal without affecting the data pollution; leak rate of ≤ 0.3%, and the correction coefficient was stable. After overall normalization and log-transformation of data SAM software was used for selection of differentially expressed miRNA. The screening condition was: FDR less than 5% and Fold change ≥ 2.0. Fluorescently labeled miRNA microarray hybridization result is shown in Figure 2.

Figure 2 fluorescently labeled miRNA microarray hybridization: Trizol method was applied to isolate total RNA before and after laryngeal cancer stem cells irradiation. After enrichment miRNA was detected and showed A260/A280 values were in the range of 1.8 to 2.1, in line with the requirements of the miRNA microarray. Such sample can be used for miRNA microarray experiments. After hybridization of labeled and purified miRNA with microarray chip, it was scanned to afford images at 543 nm channel.

Differentially expressed miRNAs of laryngeal squamous carcinoma stem cells before and after irradiation

After microarray hybridization, scanning and data analysis of two sets of miRNAs expression in laryngeal squamous carcinoma stem cells before and after irradiation, hybridization experiment was repeated twice followed by cluster analysis with Cluster3.0. Spectral clustering analysis of miRNA expression before and after irradiation showed that there were 70 miRNAs with 2 times differential expression before and after the irradiation of laryngeal cancer stem cells. There were 8 with 2-fold upregulation:hsa-miR-20b-5p; hsa-miR-197-3p; hsa-miR-338-3p; hsa-let-7f-5p; hsa-miR-3182; hsa-let-7a-5p; hsa-miR-3613-3p; hsa-miR-1973. There were 62 with 2-fold downregulation:hsa-miR-138-2-3p; hsa-miR-208b; hsa-miR-1236; hsa-miR-299-3p; hsa-miR-

1915-3p; hsa-miR-505-5p; hsa-miR-206; hsa-miR-135b-5p; hsa-miR-519e-3p; hsa-miR-519e-5p; hsa-miR-3907; hsa-miR-302e; hsa-miR-589-5p; kshv-miR-K12-12*; hsa-miR-3146; hsa-miR-629-5p; hsa-miR-4292; hsa-miR-122-3p; hsa-miR-634; ebv-miR-BART2-3p; hsa-miR-200c-3p; hsa-miR-520d-5p; hsa-miR-937; hsa-miR-620; hsa-miR-615-3p; ebv-miR-BART13; hsa-miR-3941; hsa-miR-99b-3p; hsa-miR-32-3p; hsa-miR-4258; hsa-miR-553; hsv2-miR-H7-5p; hsa-miR-3620; hsa-miR-4308; hsv2-miR-H7-3p; hsa-miR-21-3p; ebv-miR-BART6-3p; hsa-miR-361-5p; hsa-miR-625-3p; hsa-miR-23a-5p; hsa-miR-574-5p; hsa-miR-638; hsa-miR-630; hsv1-miR-H8*; ebv-miR-BART19-3p; hsa-miR-711; hsa-miR-25-5p; hsa-miRPlus-I874*; ebv-miR-BHRF1-2; kshv-miR-K12-3; hsv1-miR-H7*; hsa-miR-498; hsa-miR-300; hsv2-miR-H25; hsa-miR-1284; hsa-miR-664-3p; hsa-miR-33b-5p; hsv1-miR-H14-3p; sv40-miR-S1-5p; hsa-miR-324-5p; ebv-miR-BART18-3p; hsa-miR-877-5p.

RT-PCR validation results of differentially expressed miRNA

Poly (A) polymerase was used at the 3 'end to add tail, and then single nucleotide anchor Olig-dT primer of 5' with a 40 nt extending sequence was used for reverse transcription. 80 – 83 bp cDNA was obtained followed by Eva Green quantitative real-time PCR amplification with specific primer. This method was applied to detect the expression of some miRNA that showed differential expression before and after irradiation in chips, and 5SRNA was used for normalization. The chip results and fluorescence quantitative RT-PCR results were compared and the results showed consistent expression of differential miRNA detected from chip and quantitative RT-PCR, suggesting test results of this experiment are true and reliable (Figure3A-B).

Figure 3 A-B Some miRNA expression detected by qRT-PCR and the chip: Eva Green quantitative real-time PCR amplification was applied to detect the expression of some miRNA that showed differential expression before and after irradiation in chips. The chip results and fluorescence quantitative RT-PCR results were compared and the results showed consistent expression of differential miRNA detected from chip and quantitative RT-PCR.

Discussion

There are a variety of separation and identification of tumor stem cells such as: microspheres isolation and culture method, side population cell sorting method. The most recognized cell molecular "marker" is CDl33+ (Mangoni et al., 2012). Our group has also carried out a series of preliminary studies of cancer stem cells: successful isolation, identification and amplification of laryngeal squamous carcinoma stem cells from Hep-2 laryngeal carcinoma cell line. This study intents to further arrive at the level of gene regulation of tumor stem cells and observe the changes in the most eye-catching miRNAs. Micro RNA (miRNA) is a newly discovered short non-coding sequencing single-stranded RNA molecule in recent years which is composed of 21 to 25 nucleotides. It is the most important class of gene regulatory molecules currently known in tumor cells. The discovery of miRNA has become another significant progress and research focus of oncology research in recent years (Yu et al., 2007; Silber et al., 2008; Friedman and Jones, 2009) which including studies on the relationship between miRNAs and radiosensitivity.

Chaudhry et al. (2010) found there was differential expression of miRNA in TK6 and WTK1 cell lines (both human lymphoblastoma cells) that have different radiation resistance. The analysis showed that significantly differential expression of miRNA is closely related to different radiation resistance of TK6 and WTK1. Wang, et al. (2011) reported 12 differently expressed miRNAs in the radiotherapy sensitive and resistant non-small cell lung cancer samples. Comparing with radiotherapy resistant patients, five miRNAs (miRNA-126, miRNA-let-7a, miRNA-495, miRNA-451 and miRNA-128b) were significantly upregulated and seven miRNAs (miRNA-130a, miRNA-106b, miRNA-19b, miRNA-22, miRNA-15b, miRNA-17-5p and miRNA-21) were greatly downregulated in radiotherapy sensitive group. By further analysis, they believed that miRNA-126 promoted the cancer cells apoptosis induced by irradiation through the PI3K-Akt pathway. Furthermore, some recent studies focused on cancer radiosensitivity-modulated targets or miRNAs of cell signal transduction pathways. Shin et al. (2009) investigated the profile of miRNA expression following ionizing radiation in the human lung carcinoma cell line A549, and the expression profiles of radiation-responsive miRNAs were identified and confirmed by qRT-PCR and microarray analysis. Target prediction for radiation-responsive miRNAs suggested that their target genes could be related to apoptosis, regulation of cell cycle, and DNA damage and repair. Taken together, it is indicated that miRNA expression may be affected by radiation, and in turn involved in the regulation of radiation responses. In Josson's research (Josson et al., 2008). several miRNAs were significantly changed in prostate cancer cells in response to radiation treatment, with miR-521 downregulated and miR-34c upregulated. By altering the levels of DNA repair protein, CSA, miR-521 sensitized prostate cancer cells to radiation treatment. Lee et al. (2011) demonstrated that microRNA-7 increases radiosensitivity of human cancer cells with activated EGFR-associated signaling, and Qu's experiment (Qu et al., 2012) testified that MiR-205 determines the radioresistance of human nasopharyngeal carcinoma by directly targeting PTEN. More studies (Lal et al., 2009; Hu et al., 2010; Ng et al., 2010; Moskwa et al., 2011) revealed that miRNA can impact the radiosensitivity by modulating the expression of key proteins in DNA damage repair pathways.

Currently there is no study on relationship of miRNAs with the radiosensitivity of laryngeal cancer stem cells, which are recognized to be the source of radiation resistance as described above. Specific miRNAs may play an important regulating role in radiation-related gene of cancer stem cells.

In this study, we successfully isolated, identified and amplified laryngeal squamous carcinoma stem cells, and further studied specific changes of miRNA before

and after simulated radiotherapy. In this experiment, the currently employed oligonucleotide microarray of miRNA expression profile was selected to comparatively analyze the miRNA expression level before and after laryngeal cancer stem radiation. It was found that there were 70 miRNA with 2-fold difference in expression after laryngeal cancer stem cells radiation, eight of which were up-regulated and 62 down-regulated. The result was validated with real-time quantitative RT-PCR. Currently it is yet impossible to predict in these differentially expressed miRNA which affects the radiation sensitivity of laryngeal cancer uniquely, which is just accompany relationship, and what is the target gene of the differential expression of miRNA? These issues will be the follow-up experiments by large sample, repeated and dynamic detection of laryngeal tissue for the miRNAs expression analysis, and further clarify the biological function of miRNA with laryngeal cancer-specific differential expression.

In conclusions, in this study 70 laryngeal cancer related miRNAs with 2-fold difference in expression were screened out: eight of which were up-regulated with more than 2-fold difference in expression and 62 were down-regulated. These miRNA may be involved in the self-regulation of laryngeal squamous carcinoma stem cells radiation.

Acknowledgements

We sincerely thank Professor XT Cao for his academic help in this work. Also, we acknowledge Dr. LF Zhou for technical assistance. This work was supported by grants from the Nature Science Foundation of China (#81072495). the Science and Technology Project of Zhejiang province, China (#2009F80007; #2010C33006). We acknowledge Chemicon, Toxin Tech.and Exiqon for their pharmaceutical and industry support.

References

Avissar M, McClean MD, Kelsey KT, Marsit CJ (2009). MicroRNA expression in head and neck cancer associates with alcohol consumption and survival. *Carcinogenesis*, **30**, 2059-63.

Blazek ER, Foutch JL, Maki G (2007). Daoy medulloblastoma cells that express CD133 are radioresistant relative to CD133- cells, and the CD133+ sector is enlarged by hypoxia, Int J Radiat. *Oncol Biol Phys*, **67**, l-5.

Chaudhry MA, Sachdeva H, Omaruddin RA (2010). Radiation-induced micro-RNA modulation in glioblastoma ceils differing in DNA repair pathways, *DNA Cell Biol*, **29**, 553-61.

Friedman JM, Jones PA (2009). MicroRNAs: critical mediators of differentiation, development and disease. *Swiss Med Wkly*, **139**, 466-72.

Ghotra VP, Puigvert JC, Danen EH (2009). The cancer stem cell microenvironment and anti-cancer therapy. *Int J Radiat Biol*, **85**, 955-62.

Hambardzumyan D, Squatrito M, Holland EC (2006). Radiation resistance and stem-like cells in brain tumors. *Cancer Cell*, **10**, 454-6.

Hu H, Du L, Nagabayashi G, et al (2010). ATM is down-regulated by N-Myc-regulated microRNA-421. *Proc Natl Acad Sci USA*, **107**, 1506-11.

Hime GR, Somers WG (2009). Micro-RNA mediated regulation of proliferation, self-renewal and differentiation of mammalian stem cells. *Cell Adh Migr*, **3**, 425-32.

Josson S, Sung SY, Lao K, et al (2008). Radiation modulation of microRNA in prostate cancer cell lines. *Prostate*, **68**, 1599-606.

Kang MK, Har BI, Ko MH, et al (2008). Potential identity of multi-potential cancer stem-1ike subpopulation after radiation of cultured brain glioma. *BMC Neurosci*, **9**, 15.

Lal A, Pan Y, Navarro F, et al (2009). MiR-24-mediated downregutation of H2AX suppresses DNA repair in terminally differentiated blood cells. *Nat Struct Mol Biol*, **16**, 492-8.

Lee KM, Choi EJ, Kim IA (2011). MicroRNA-7 increases radiosensitivity of human cancer cells with activated EGFR-associated signaling. *Radiother Oncol*, **101**, 171-6.

Mangoni M, Livi L, Biti G, et al (2012). Stem cell tracking: toward clinical application in oncology? *Tumori*, **98**, 535-42.

Moskwa P, Buffa FM, Pan Y, et al (2011). MiR-182-mediated downregulation of BRCAl impacts DNA repair and sensitivity to PARP inhibitors, *Mol Cell*, **41**, 210-20.

Mueller DW, Bosserhoff AK (2009). Role of miRNAs in the progression of malignant melanoma. *Br J Cancer*, **101**, 551-6.

Ng WL, Yan D, Zhang X, et al (2010). Over-expression of miR-100 is responsible for the low-expression of ATM in the human glioma cell line: M059J. *DNA Repair*, **9**, 1170-5.

Qu C, Liang Z, Huang J, et al (2012). MiR-205 determines the radioresistance of human nasopharyngeal carcinoma by directly targeting PTEN. *Cell Cycle*, **11**, 785-96.

Schetter AJ, Leung SY, Sohn JJ (2008). MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA*, **299**, 425-36.

Shin S, Cha Hj, Lee EM, et al (2009). Alteration of miRNA profiles by ionizing radiation in A549 human non-small cell lung cancer cells. *Int J Oncol*, **35**, 81-6.

Silber J, Lim DA, Petritsch C, et al (2008). MiR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. *BMC Med*, **6**, 14.

Valeri N, Croce CM, Fabbri M (2009). Pathogenetic and clinical relevance of microRNAs in colorectal cancer. *Cancer Genomics Proteomics*, **6**, 195-204.

Wang XC, Du LQ, Tian LL, et al (2011). Expression and function of miRNA in postoperative radiotherapy sensitive and resistant patients of non-small cell lung cancer. *Lung Cancer*, **72**, 92-9.

Yu F, Yao H, Zhu P, et al (2007). Let-7 regulates self renewal and tumorigenicity of breast cancer cells. *Cell*, **131**, 1109-23.