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Regulatory Role of *Doxycycline* on Expression of *mi-RNA* in Tumorigenesis Pathway of Colorectal Cancer

Neha Nanda[1,2], Devinder Kumar Dhawan[2], Safrun Mahmood[1*]

[1]Department of Experimental Medicine and Biotechnology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India

[2]Department of Biophysics, Panjab University, Chandigarh, India

[1]*Correspondence to Dr. Safrun Mahmood, Geneticist, Department of Experimental Medicine and Biotechnology, Research Block-B, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh-160012, India

Abstract: ***Downregulation of mir-143 and mir-145 has been demonstrated as a common feature of colon cancer. Several studies have shown that upregulation of mir-143 and mir-145 may be a good aspect for therapeutic purposes in the treatment of colon cancer. Doxycycline an anti-tumorigenic agent, was studied to determine the modulation of mir-143 and mir-145 expression in DMH-induced colon cancer in rats. We have found that doxycycline (DMH/DOX) unexpectedly increased aberrant crypt foci (ACFs) in DMH treated rats and caused progression of colonic tumor growth. Further analysis of mir-143 and mir-145 expression in control and treated rats, showed that compared to controls the expression of mir-143 and mir-145 was diminished in DMH/DOX treated rats. The data suggest that Doxycycline enhances tumorous growth in colon of DMH treated rats and does not modulate the expression of mir-143 and mir-145 in colon cancer.***

Keywords: Doxycycline; mir-143; mir-145; ACFs; Colon cancer

1. Introduction

MicroRNAs (miRNAs) are highly conserved, small (18–25 nucleotide in length), single-stranded non-coding RNAs (Ambros, 2004). miRNAs regulate gene expression and translation of protein-coding genes in the mammalian genome by acting on messenger RNA (mRNA) of target genes and inducing degradation of mRNA or inhibition of translation process (Bartel, 2004). The involvement of miRNAs in the development of malignant neoplastic disease was first demonstrated by Calin et al (2002). Till now, the role of miRNAs has been intensively investigated in multiple human diseases (Mendell and Olson, 2012), and their involvement in dysregulation of mRNA expression, has been shown in almost all types of human cancers (Lu et al, 2005; Volinia et al 2006). Some of these miRNAs function as oncogenes or tumor-suppressors, depending upon the inhibition of target genes. mir-143 and mir-145 are kind of these miRNAs which are downregulated in various cancers such as lung cancer, breast cancer and colon cancer (Gao et al, 2010; Bockmeyer et al, 2011).

Colorectal cancer is the third most common cancer and one of the leading causes of death worldwide, being responsible for about 10% of total cancer-related mortality (Jemal et al 2008). Despite decades of research and the current development of novel therapeutic approaches, the 5 year survival rate for colon cancer at distant stage is 12%. Colon carcinogenesis is a multistage process with accumulation of genetic and epigenetic alterations in normal colon epithelium, which develop a a well-defined adenoma-carcinoma sequence (Fearon, Vogelstein 1990).

Several studies have shown the chemotherapeutic potential of various tetracyclines. One of the effective tetracycline is doxycycline, which has been used for therapeutic purposes in various diseases. Some studies have shown that doxycycline exhibits potent chemotherapeutic activity in colon cancer cell lines, alone and with combination of other therapeutic compounds (Onoda et al, 2006).

In the present study, we have developed an animal model of colon cancer in two different stages, adenoma and adenocarcinoma. Further, we desired to evaluate the effectiveness of doxycycline as a chemotherapeutic agent both on 10 and 20 weeks DMH treatments. To our surprise, we found that doxycycline treatment caused ACFs formation and increased tumor growth in 10 weeks DMH-treated rat colon. Also, we have studied the expression of mir-143 and mir-145 in different stages of colon carcinogenesis and with doxycycline treatment to find out that whether doxycycline modulate the expression of these miRs or not.

2. Material and Methods

Animals

All animal studies were performed in accordance with protocols approved by the Institutional Animal Ethics Committee. Healthy, specific pathogen free Sprague Dawley rats with body weight ranging from 120 to 150g were kept in polypropylene cages under hygienic conditions. Prior to start of the experiments, the animals were fed a stock pellet diet and water ad libitum for one week to acclimatize to the laboratory conditions. After acclimatization, rats were divided into four groups of 24 rats in each group. Group I received weekly subcutaneous injections of normal saline (Controls), while Group II rats were injected with 1, 2

dimethylhydrazine (DMH) (Sigma), subcutaneously (s.c.) at a dosage of 30mg/kg body weight (Soler et al, 1999), weekly for 10 weeks (Adenoma) and 20 weeks (carcinoma). DMH was freshly prepared in normal saline, pH adjusted to 7.0 using dilute NaOH solution. After 10 weeks of treatment, all the rats developed adenomas while colonic tumors were developed after 20 weeks of treatment and the size of tumors was followed by direct measurement. Group III received daily intraperitoneal injections of DOX followed by 10 weeks of DMH treatment at a dose of 10 or 20 mg/kg body weight (DMH/DOX-10, DMH/DOX-20) in normal saline for 15 days.

Methylene blue staining

Colons were quickly excised, flushed with saline, slit open longitudinallyfrom the cecum to anus and fixed flat between two pieces of filter paper in 10% phosphate-buffered formalin. After fixation for at least 24 h at 4°C, all colons were stained with 0.2% methylene blue for 3–5 min and then examined for ACFs by light microscopy.

miRNA quantification

miRNA from colonic tissues, was isolated with the mirVana miRNA Isolation Kit (Ambion). Quantification of the mature form of miRNAs was performed with TaqMan MicroRNA Assay Kit, in accordance with the manufacturer's instructions (Applied Biosystems). The U6 small nuclear RNA was used as an internal control.
Primer sequences were as follows:
rno-miR-143-3p 5'- UGAGAUGAAGCACUGUAGCUCA-3'
rno-miR-145-5p 5'-
GUCCAGUUUUCCCAGGAAUCCCU- 3'

3. Statistical Methods

Statistical analysis between the groups was done by ANOVA. Values with $P<0.05$ were considered statistically significant.

4. Results & Discussion

We used the animal model of colon cancer in Sprague Dawley rats (Group-II) by injecting DMH for two time durations i.e. 10 weeks and 20 weeks. After sacrificing, we found that 10 weeks DMH treated rats showed formation of aberrant crypt foci (ACFs) in colonic mucosa, whereas increased number of intensely stained ACFs was found in 20 weeks DMH treated ratrs. However, no sign of ACFs was observed in colon of normal rats (Figure 1a,b & c). These findings are similar to those reported by Prasad et al (2014), suggesting that colonic mucosa of DMH treated rats showed crypt abscess, aberrant crypt foci, and nuclear enlargement as compared to colon of control rats.

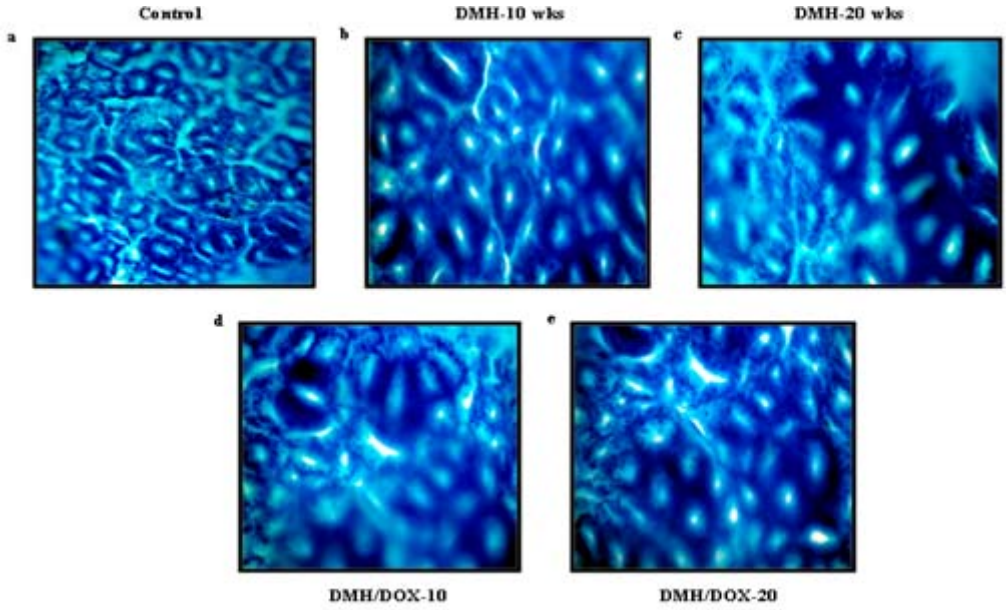

Figure 1: Formation of Aberrant crypt foci (ACFs) in the colons of Sprague Dawley rats. Methylene blue stain of colons from control and treated rats (n=24); original magnification ×10 & ×20.

After developing the colon cancer model in two different stages, adenoma and adenocarcinoma, we checked the chemotherapeutic efficacy of doxycycline in both 10 and 20 weeks DMH-treated rats. For this, we employed new animals (Group-III) and treated these animals with DMH for 10 weeks to develop adenoma, after that we injected doxycycline with two different doses viz 10 and 20 mg/kg body wt intraperitoneally for 15 days. The total duration of treatment was 10 weeks 15 days or nearly 12 weeks. Thereafter, rats were sacrificed. Surprisingly, we found that 15 days doxycycline treatment with both the doses caused increased number of methylene blue stained ACFs with nuclear enlargement in colon of all the 10 weeks DMH-treated rats (Figure 1d & e). With these unusual findings after doxycycline treatment observed in 10 weeks DMH treated rats, we could not proceed with 20 weeks DMH treatment. However, Wargovich et al (2000) reported that tetracyclines are strong inhibitors of carcinogen-iduced ACFs in the colon.

Further, we have analysed the expression of mir-143 and mir-145microRNAs in the colonic tissues. We observed

significantly reduced expression of mir-143 in 10 weeks DMH-treated rats i.e ~0.7 fold and 20 weeks DMH-treated rats i.e ~0.98 fold as compared to controls. A significant downregulation of mir-143 by ~0.96 fold in DMH/DOX-10 and ~0.98 fold in DMH/DOX-20 treated animals as compared to controls, was observed (Figure 2).

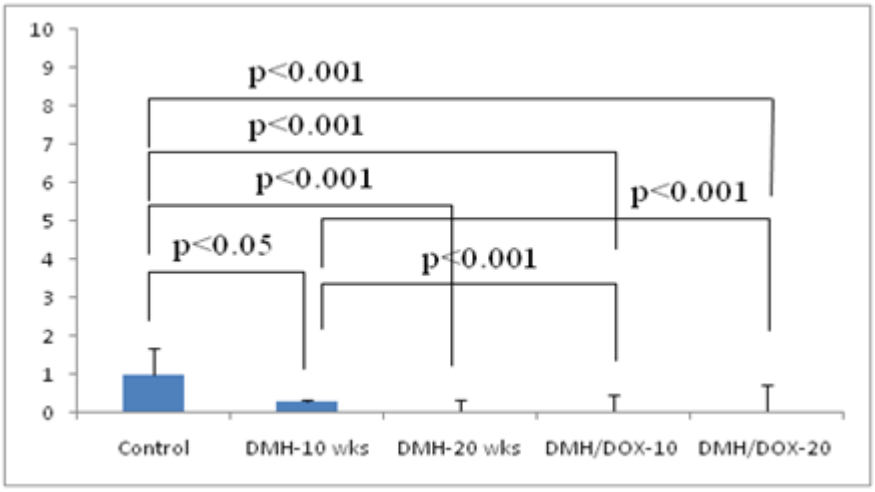

Figure 2: mir-143 expression was downregulated in colon cancer model and doxycycline treated rats.

We also checked the expression of mir-145 in these animals and found similar results. The expression of mir-145 was downregulated in colonic tissues of 10 weeks DMH-treated rats i.e ~0.9 fold and 20 weeks DMH-treated rats i.e ~0.99 fold as compared to controls. After doxycycline treatment with 10mg/kg body wt, we demonstrated a significant reduction of mir-145 by ~0.9 fold in DMH/DOX-10 treated rats and with 20mg/kg body wt, a significant reduction in mir-145 by ~0.997 fold in DMH/DOX-20 treated rats as compared to controls (Figure 3).

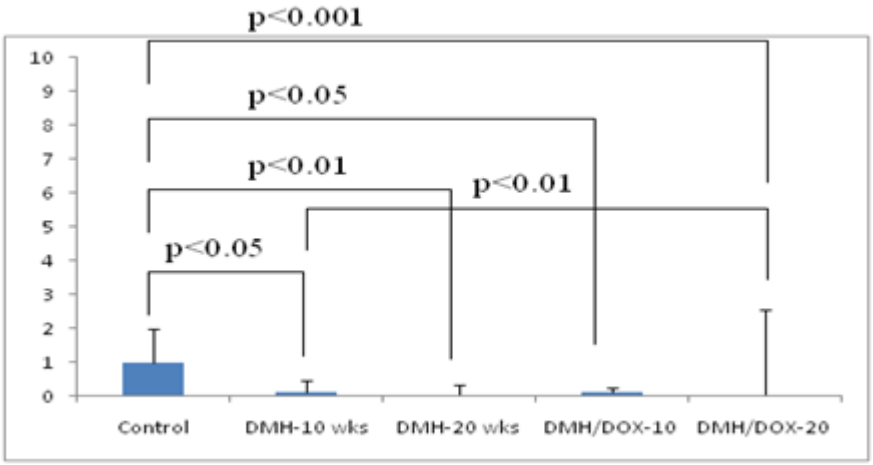

Figure 3: mir-145 expression was downregulated in DMH-induced colon cancer and doxycycline treated rats

The present study demonstrates that mir-143 and mir-145 was significantly downregulated in colon cancer. These findings are supported by several studies, Pagliuca et al (2013) have shown that mir-143-145 cluster expression was downregulated in colon cancer cell lines (HCT-116, SW480 and HT-29) as compared to normal colonic epithelium (NCE) cells. Similarly, Kent et al (2013) have demonstrated that in colorectal cancer cell lines, the miR-143/145 cluster is repressed by RREB1 gene which is a downstream of constitutively active KRAS. In addition, these findings clearly suggest that doxycycline does not play any role in modulation of mir-143 and mir-145 expression in colon cancer model. To the best of our knowledge to date, there are no reports in the literature regarding the effects of doxycycline on expression of mir-143 and mir-145 under in vivo conditions in an animal model of colon carcinogenesis.

5. Conclusion

In conclusion, present findings suggest that doxycycline caused increased number of aberrant crypt foci and enhances tumor growth in colon of DMH treated rats. Also, doxycycline does not modulate mir-143 and mir-145 expression in colon cancer model, rather it diminished the expression of these miRs.

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