Int J Clin Exp Pathol 2018;11(8):3961-3968
www.ijcep.com /ISSN:1936-2625/IJCEP0010619

Original Article

Intermedin promotes hepatic carcinoma cell proliferation through upregulation of miR-155

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Received May 24, 2015; Accepted June 29, 2015; Epub August 1, 2018; Published August 15, 2018

Abstract: Objective: MicroRNAs (miRNAs) plays an important role in the development of malignant carcinoma. The small peptide intermedin (IMD) can promote hepatic carcinoma cell proliferation. The aim of the present study is to examine the effect of miR-155 on IMD-stimulated hepatic carcinoma cell proliferation. Methods: Proliferation of hepatic carcinoma SMMC7721 cells was detected by CCK-8, expression of proliferating cell nuclear antigen (PCNA) and miR-155 was detected by real-time PCR. Results: We found that IMD promotes the proliferation of SMMC7721 cells in a time and dose-dependent manner. IMD can upregulate the expression of miR-155, and blocking of miR-155 can inhibit the IMD-induced SMMC7721 cell proliferation to some extent. Conclusion: This study demonstrated that IMD can promote the proliferation of human hepatic carcinoma cell line SMMC7721 cells through upregulation of miR-155. This study may contribute to hepatic cancer prevention and therapy.

Keywords: Intermedin, hepatic carcinoma, proliferation, miR-155

Introduction

MicroRNAs (miRNAs) is a recently discovered single-stranded non-coding small molecules RNA with length of about 22 nucleotide, which constitute a large class of widely found gene regulator in plant and animal cells, and it can regulate post-transcriptional gene expression through completely or incompletely binding with target gene [1-3]. The abnormal miRNAs expression is associate with variety of tumors, and different kinds of miRNAs have two different roles of carcinogenic and tumor inhibition [4, 5]. Recent studies have found that miR-155 expression was upregulated in breast cancer, pancreatic cancer, lung cancer and other malignant tumors, and plays an important regulatory role in terms of its biological behavior [6-8].

Primary hepatocellular carcinoma is the most common malignant tumors worldwide, and its incidence ranked No. 3 in malignant tumors with high degree of malignancy and low five-year survival rate of about 30%, and there is no effective treatment yet. Study on the mechanism of hepatocellular carcinoma occurrence, development and metastasis, and to explore the effective therapeutic methods is a hot topic in hepatocellular carcinoma study, and the proliferative activity of hepatic carcinoma cell is directly related with tumor occurrence, invasion and metastasis. Intermedin (intermedin, IMD) is a peptide found in 2004 [9, 10], it belongs to calcitonin gene related peptide (CGRP) superfamily and widely distributed in the body, and CGRP superfamily members play an important regulation effect in ion balance, nerve transmission, glucose metabolism and cardiovascular and endocrine homeostasis, and other physiological processes, thus get more and more attention in tumor development. Studies found that the IMD expression in adrenocortical tumors [11] and colorectal cancer [12] was higher than that in the normal control tissues, and IMD can promote angiogenesis [13], suggesting that IMD may participate in the development and progression of tumors. This paper explored whether IMD plays a role in the occurrence and development of hepatic carcinoma through regulating miRNAs.

Materials and methods

Recombinant human intermedin (IMD1-53) was purchased from America Phoenix Pharmaceuti-

cals Company. CCK-8 cell proliferation assay kit was purchased from the Dojindo Molecular Technologies, Inc.; RNA extraction and reverse transcription kits and Luciferase reporter gene assay kit were purchased from Promega Company, USA; Taq enzyme was purchased from Tiangen Biotech (Beijing) Co. Ltd.; Evergreen fluorescent dye was purchased from American Biotium Company; miR155 antisense oligonucleotide ASO-miR-155 was purchased from Gene Pharma Company; Lipofectmine 2000 transfection reagents were purchased from Invitrogen Company, USA.

Cell proliferation detected with CCK-8

SMMC7721 cells in the logarithmic growth phase were inoculated in flat-bottomed 96-well plates with 1×10^4/100 μl, after 12 hours, replaced with the culture medium with different concentrations of IMD1-53 and RPMI 1640, or added with ASO-miR-155, and the complete culture medium without IMD and RPMI1640 was used as control, taken the plate at 48 hours and added with 10 μL CCK-8 in each well, then placed in the incubator for 1-4 hours, and microplate reader was used to measure the absorbance value of 450 nm wavelength [14].

RNA extraction

Trizol reagents of Promega Company was used: the cells were aspirated from the culture medium and washed with PBS, then added 1 ml RNAtrip reagent and repeatedly triturated for 30 times; transferred into the EP tube and placed at room temperature for 10 minutes; added 0.2 ml chloroform and dramatic reversal mixing for 15 seconds, placed at room temperature for 5 minutes; centrifuged at 12000 g ×15 min with the temperature of 4°C; carefully aspirate the upper aqueous phase and transferred to a new EP tube, then added the equal volume of isopropanol and mix well, precipitation for 2 hours at -70°C; centrifuged at 12000 g ×10 min with the temperature of 4°C. The supernatant was discarded, precipitated and washed with 1ml ice-cold ethanol with the concentration of 75%, centrifuged at 12000 g ×10 min with the temperature of 4°C; abandoned the supernatant, and washed the droplet attached to the wall with pipette. After transparent appeared around the precipitation and whitish in the center, added RNA enzyme and dissolved with water and quantitative.

Stem-loop primer real-time quantitative PCR

Reverse transcription reaction was 20 ml reaction system, 2 mg RNA starting, reverse transcription kit of Promega Company was used for first strand cDNA synthesis. Real-time quantitative PCR was 25 ml reaction system; 1 ml reverse transcription reaction mixture was used as template. Amplification conditions were as follows: pre-degeneration for 1 minutes at 94°C, degeneration for 5 seconds at 94°C, annealing for 30 seconds at 58°C, extension for 30 seconds at 72°C, then extension for 5 minutes at 72°C, a total of 40 circulation. Stratagene Mx3000 software was used for analysis. The reverse transcription and PCR primers are as follows:

Primers		Sequence (5'-3')
miR-155	RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACCCCTAT
	Forward	ACACTCCAGCTGGGTTAATGCTAATCGTGAT
	Reverse	GTGCAGGGTCCGAGGT
U6	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATA
	Forward	CTCGCTTCGGCAGCACATA
	Reverse	GTGCAGGGTCCGAGGT
PCNA	Forward	TGTTGGAGGCACTCAAGGAC
	Reverse	TCATTGCCGGCGCATTTTAG
β-actin	Forward	ATCTGGCACCACACCTTC
	Reverse	AGCCAGGTCCAGACGCA

Statistical analysis

All results were presented as mean ± standard error (mean ± SD); Prism software was used for analysis and graphing. One way analysis of variance was conducted for multi-group comparison, Student-Newman-Keuls was used for further analysis and comparison between groups. T-test was used to compare the results between groups, and $P<0.05$ was considered significantly different.

Results

IMD can promote SMMC7721 cell proliferation

We firstly explored the effect of IMD on SMMC7721 cell proliferation. CCK-8 test showed that intervention with IMD (5-100 nM) for 48 hours could significantly promote SMMC7721 cell proliferation (*$P<0.05$, **Figure 1**). IMD also can significantly upregulate the expression of proliferating cell nuclear antigen (PCNA) (**Figure 2**).

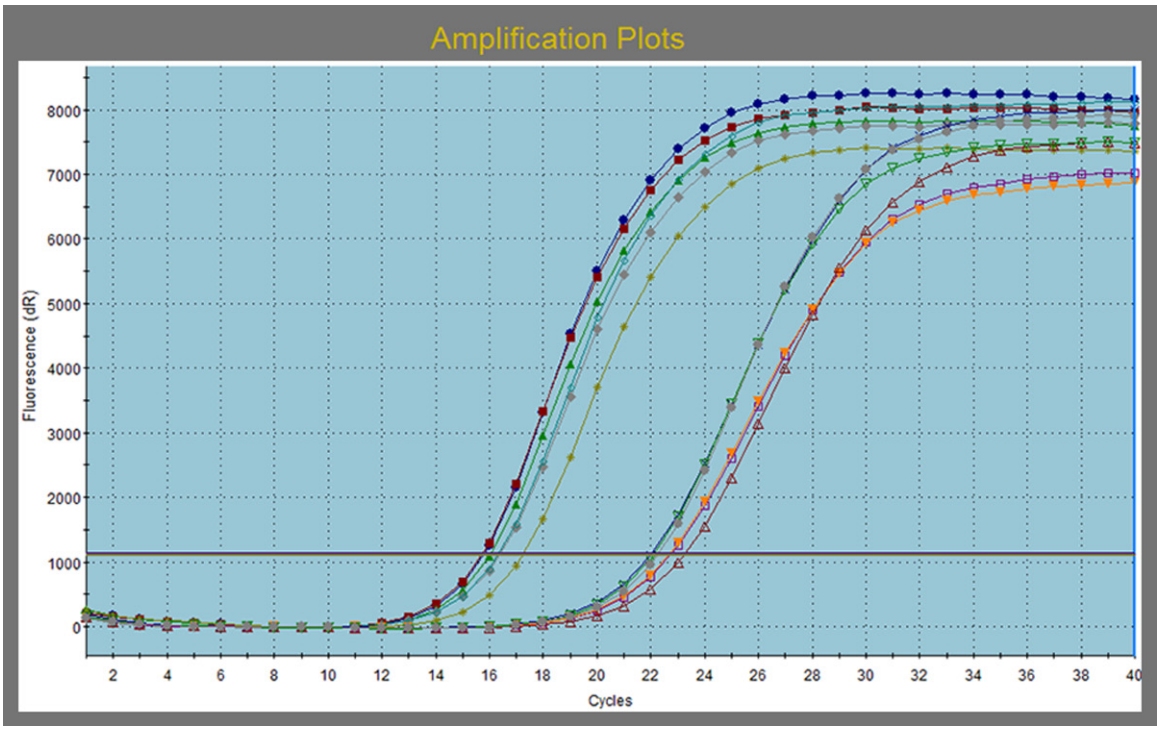

Figure 1. Intermedin (IMD) promotes SMMC7721 cell proliferation. SMMC7721 cells were treated with indicating dosages of IMD for 48 h and cell viability was detected with CCK-8 assay. Data are means ± SEM from 3 separate experiments. *P<0.05 versus IMD-untreated cells.

IMD can upregulate the expression of miR55 of AMMC7721 cells

We further explored the possible mechanism of IMD in promoting SMMC7721 cell proliferation. A variety of factors involved in tumor cells migration and invasion, and the endogenous non-coding small molecule RNA (microRNA), the research focus in recent years, plays an important role in regulating gene transcription and protein translation, and miR-155 can promote tumor proliferation. We examined the impact of IMD on the expression of miR-155, and found that IMD (50 nM) could significantly upregulate the expression of miR-155 (**Figure 3**).

Inhibition of miR-155 can inhibit IMD-induced SMMC7721 cell proliferation to some extent

The miR155 antisense oligonucleotide ASO-miR-155 (100 nM) transfected with SMMC7721 cells for 48 hours can significantly inhibit IMD (50 nM)-induced SMMC7721 cell proliferation (**Figure 4**) and PCNA expression (**Figure 5**), so IMD could promote SMMC7721 cell proliferation through upregulating miR155.

Discussion

This study firstly confirmed that human IMD could promote hepatic carcinoma SMMC7721 cell proliferation by upregulating the expression of miR155. The basses are as follows: (1) IMD-induced hepatic carcinoma SMMC7721 cells can directly cause cell proliferation and upregulate the level of PCNA expression; (2) IMD can upregulate the expression of miR-155 in SMMC7721 cells; (3) Blocking miR155 with miR155 antisense oligonucleotide ASO-miR-155 can inhibit IMD-induced SMMC7721 cell proliferation and the increasing of PCNA expression to some extent.

IMD belongs to CGRP superfamily, the members of this family are small peptides, including adrenomedullin (AM), CGRP and islet amyloid polypeptide. IMD is recently discovered CGRP superfamily polypeptide, it was firstly discovered in 2004 in teleost fish [10], and then this active peptide has been found in mammals and human cDNA clones [9]. Both lower animals, mammals and humans IMD has highly conserved sequences. Human IMD gene is located

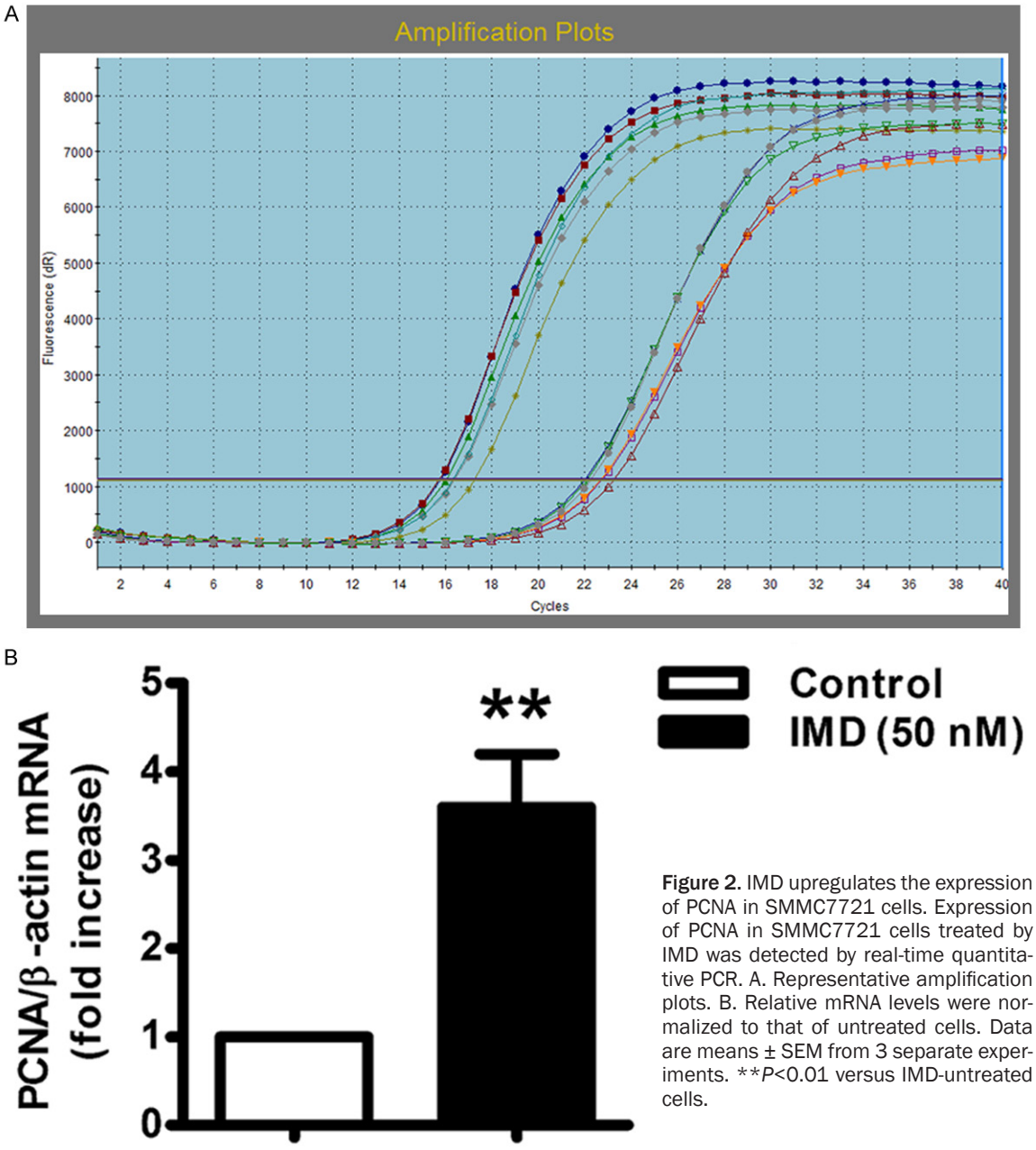

Figure 2. IMD upregulates the expression of PCNA in SMMC7721 cells. Expression of PCNA in SMMC7721 cells treated by IMD was detected by real-time quantitative PCR. A. Representative amplification plots. B. Relative mRNA levels were normalized to that of untreated cells. Data are means ± SEM from 3 separate experiments. ***P*<0.01 versus IMD-untreated cells.

on human chromosome 22, and the IMD precursor protein (prepro-IMD) is encoded 148 amino acid residues, and there is N-terminal intramolecular cyclization, and connected with S-S bond. Prepro-IMD has multiple protease cleavage sites, and can be cut into different active fragments in the body [15, 16]: IMD_{1-47}, IMD_{8-47} and IMD_{1-53}. IMD is widely distributed and mainly distributed in the submandibular gland, kidney, stomach and mesentery, followed by lung, pancreas, pituitary, intestine, spleen, thyroid and ovarian [9]; as an new protective factor of endogenous cardiovascular and kidney, IMD has important physiological and pathological role in homeostatic regulation and central nervous regulation, as well as the hypertension, myocardial ischemia, heart failure and renal failure process. Although, the effect of the same family member AM in tumor occurrence is widely researched, the effect of IMD in tumor, especially in the occurrence and development of hepatocellular carcinoma, is still unclear. The study found that the expressions of IMD in adrenocortical tumor, colorectal

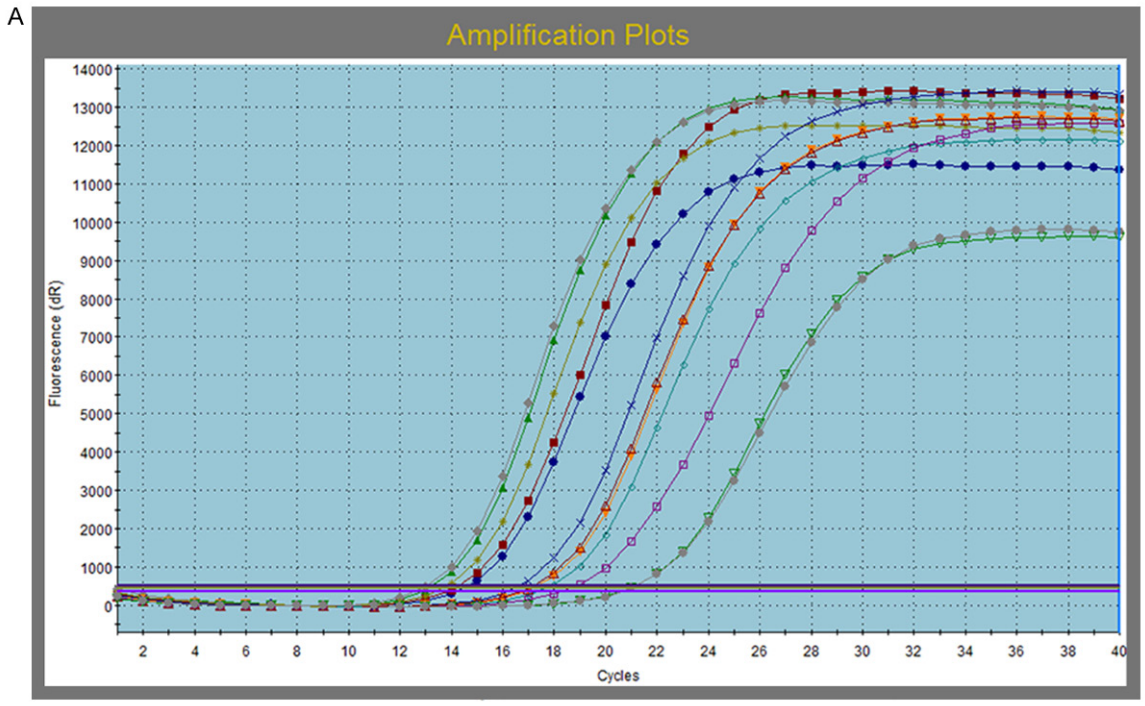

Figure 3. IMD upregulates the expression of miR-155 in SMMC7721 cells.Expression of miR-155 in SMMC7721 cells treated by IMD was detected by stem-loop real-time quantitative PCR. A. Representative amplification plots. B. Relative mRNA levels were normalized to that of untreated cells. Data are means ± SEM from 3 separate experiments. $^{**}P<0.01$ versus IMD-untreated cells.

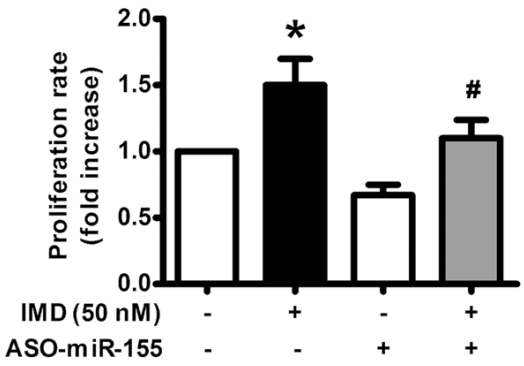

Figure 4. Blockage of miR-155 attenuates IMD-induced SMMC7721 cell proliferation. Cells were pre-treated with or without a miR-155-specific antisense oligonucleotide (ASO-miR-155) and then incubated in the presence of IMD. Cell viability was detected with CCK-8 assay. Data are means ± SEM from 3 separate experiments. $^{*}P<0.05$ versus IMD-untreated cells, $^{\#}P<0.05$ versus IMD treatment alone.

cancer, breast cancer and pancreatic cancer were higher than that in the normal control group. Our study showed that IMD can directly promote SMMC7721 cell proliferation (**Figure 1**), meanwhile, it can upregulate the expression level of the important marker PCNA in G1/S phase (**Figure 2**), and this result is consistent with the research results of Guo et al [17].

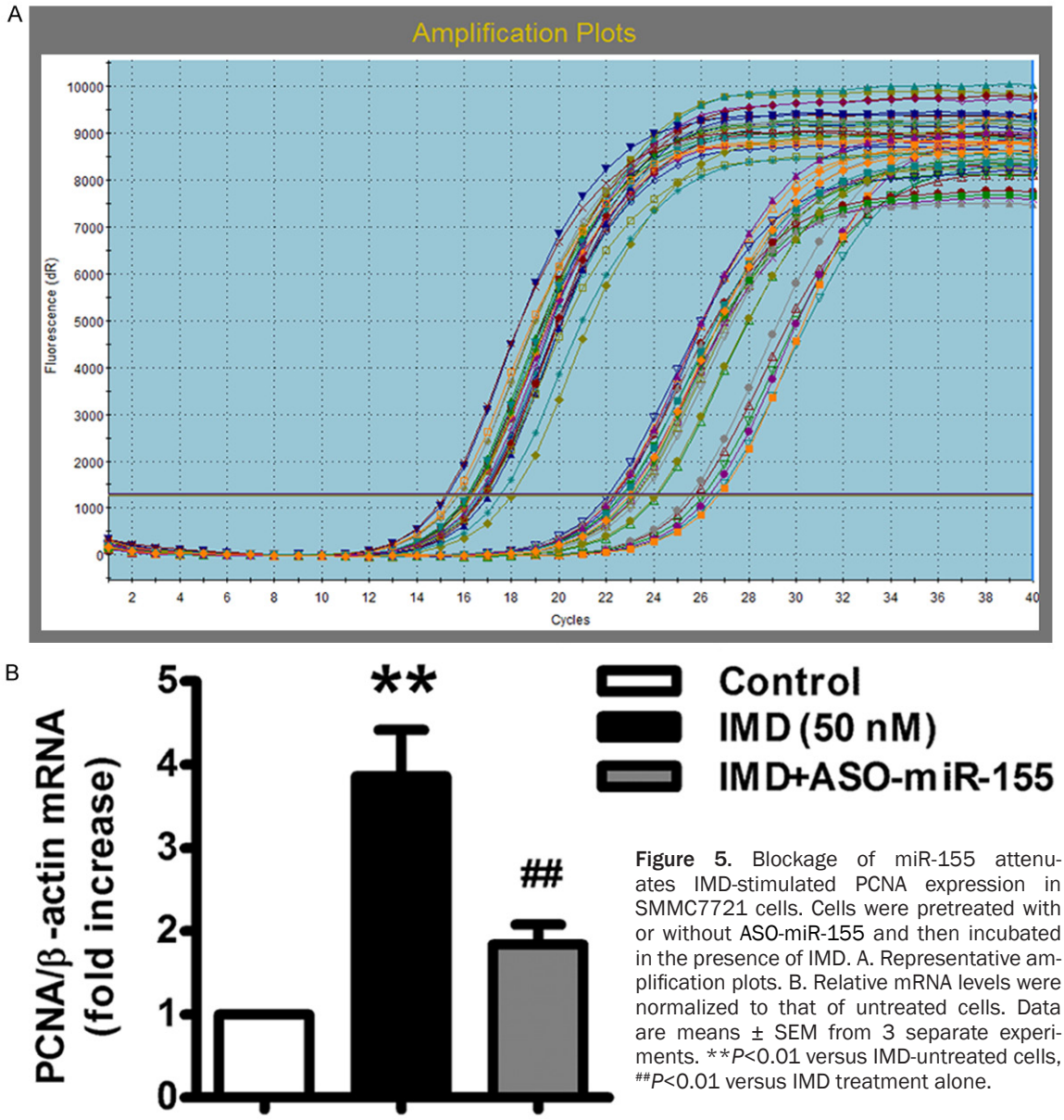

Figure 5. Blockage of miR-155 attenuates IMD-stimulated PCNA expression in SMMC7721 cells. Cells were pretreated with or without ASO-miR-155 and then incubated in the presence of IMD. A. Representative amplification plots. B. Relative mRNA levels were normalized to that of untreated cells. Data are means ± SEM from 3 separate experiments. $^{**}P<0.01$ versus IMD-untreated cells, $^{\#\#}P<0.01$ versus IMD treatment alone.

miRNA is same with protein coding genes which has important biological functions, many miRNA exhibited strong tumorigenicity and tumor suppression effect. For the function, cancer miRNAs has the effect in promote cell proliferation, inhibiting apoptosis and immune cell development, and controling cell cycle, and tumor suppressor miRNAs has the effect in inhibiting cell growth and apoptosis, and and promoting invasion. MiR-155 is located on chromosome 21q21, and conserved regional encoding with highly conserved region of the third exon gene of B cells integration cluster [18]. The expression level of miR-155 was increased in Hodgkin's lymphoma, non-Hodgkin lymphoma and breast cancer cells [6, 19], and the over expression of miR-155 in breast cancer can promote survival and proliferation of breast cancer and reduce sensitivity of breast cancer to chemotherapy drug through inhibiting forkhead box protein 03 (FOX03) expression [20], indicating that miR-155 is closely related with the occurrence and development of tumor, and it can be considered as tumor miR. In this study, IMD can significantly upregualte miR-155 expression, thus miR-155 may be involved in the regulation of IMD on proliferation of hepatocellular carcinoma cells.

Further study showed that after blocking the miR-155 action by antisense oligonucleotides, the effect of IMD in promoting cell proliferation and upregulating PCNA was masked significantly, indicating that IMD can promote hepatoma carcinoma cell proliferation and migration through stimulating tumor miRNA-miR-155. However, the upregulation mechanism of IMD on miR-155 need to be further researched.

In conclusion, this study found that as a member of CGRP superfamily, IMD, can promote hepatoma carcinoma cell proliferation through upregulating miR-155, which provides a new idea for the treatment of liver cancer. IMD antagonists may be used as one of anti cancer treatment methods.

Acknowledgements

This paper is funded by the Project of Shenyang Science and Technology Bureau, No. F12-193-9-56.

Disclosure of conflict of interest

None.

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References

[1] Zeng Y. Principles of micro-RNA production and maturation. Oncogene 2006; 25: 6156-6162.

[2] Kloosterman WP and Plasterk RH. The diverse functions of microRNAs in animal development and disease. Dev Cell 2006; 11: 441-450.

[3] Sayed D and Abdellatif M. MicroRNAs in development and disease. Physiol Rev 2011; 91: 827-887.

[4] Croce CM. Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet 2009; 10: 704-714.

[5] Tricoli JV and Jacobson JW. MicroRNA: Potential for Cancer Detection, Diagnosis, and Prognosis. Cancer Res 2007; 67: 4553-4555.

[6] Jiang S, Zhang HW, Lu MH, He XH, Li Y, Gu H, Liu MF and Wang ED. MicroRNA-155 functions as an OncomiR in breast cancer by targeting the suppressor of cytokine signaling 1 gene. Cancer Res 2010; 70: 3119-3127.

[7] Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, Stephens RM, Okamoto A, Yokota J, Tanaka T, Calin GA, Liu CG, Croce CM and Harris CC. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 2006; 9: 189-198.

[8] Bloomston M, Frankel WL, Petrocca F, Volinia S, Alder H, Hagan JP, Liu CG, Bhatt D, Taccioli C and Croce CM. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. JAMA 2007; 297: 1901-1908.

[9] Takei Y, Inoue K, Ogoshi M, Kawahara T, Bannai H and Miyano S. Identification of novel adrenomedullin in mammals: a potent cardiovascular and renal regulator. FEBS Lett 2004; 556: 53-58.

[10] Roh J, Chang CL, Bhalla A, Klein C and Hsu SY. Intermedin is a calcitonin/calcitonin gene-related peptide family peptide acting through the calcitonin receptor-like receptor/receptor activity-modifying protein receptor complexes. J Biol Chem 2004; 279: 7264-7274.

[11] Morimoto R, Satoh F, Murakami O, Hirose T, Totsune K, Imai Y, Arai Y, Suzuki T, Sasano H, Ito S and Takahashi K. Expression of adrenomedullin 2/intermedin in human adrenal tumors and attached non-neoplastic adrenal tissues. J Endocrinol 2008; 198: 175-183.

[12] Hikosaka T, Tsuruda T, Nagata S, Kuwasako K, Tsuchiya K, Hoshiko S, Inatsu H, Chijiiwa K and Kitamura K. Adrenomedullin production is increased in colorectal adenocarcinomas; its relation to matrix metalloproteinase-9. Peptides 2011; 32: 1825-1831.

[13] Hikosaka T, Tsuruda T, Nagata S, Kuwasako K, Tsuchiya K, Hoshiko S, Inatsu H, Chijiiwa K and Kitamura K. Intermedin is a new angiogenic growth factor. Am J Physiol Heart Circ Physiol 2009; 297: H1040-1047.

[14] Xu Y, Pang X, Dong M, Wen F and Zhang Y. Nesfatin-1 inhibits ovarian epithelial carcinoma cell proliferation in vitro. Biochem Biophys Res Commun 2013; 440: 467-472.

[15] Ren YS, Yang JH, Zhang J, Pan CS, Yang J, Zhao J, Pang YZ, Tang CS and Qi YF. Intermedin 1-53 in central nervous system elevates arterial blood pressure in rats. Peptides 2006; 27: 74-79.

[16] Yang JH, Jia YX, Pan CS, Zhao J, Ouyang M, Yang J, Chang JK, Tang CS and Qi YF. Effects of intermedin (1-53) on cardiac function and ischemia/reperfusion injury in isolated rat hearts. Biochem Biophys Res Commun 2005; 327: 713-719.

[17] Guo X, Schmitz JC, Kenney BC, Uchio EM, Kulkarni S and Cha CH. Intermedin is overexpressed in hepatocellular carcinoma and regulates cell proliferation and survival. Cancer Sci 2012; 103: 1474-1480.

[18] Xiang X, Zhuang X, Ju S, Zhang S, Jiang H, Mu J, Zhang L, Miller D, Grizzle W and Zhang HG. miR-155 promotes macroscopic tumor formation yet inhibits tumor dissemination from mammary fat pads to the lung by preventing EMT. Oncogene 2011; 30: 3440-3453.

[19] Eis PS, Tam W, Sun L, Chadburn A, Li Z, Gomez MF, Lund E and Dahlberg JE. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. Proc Natl Acad Sci U S A 2005; 102: 3627-3632.

[20] Kong W, He L, Coppola M, Guo J, Esposito NN, Coppola D and Cheng JQ. MicroRNA-155 regulates cell survival, growth, and chemosensitivity by targeting FOXO3a in breast cancer. J Biol Chem 2010; 285: 17869-17879.