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Short communication

The polysaccharides from fermented *Ganoderma lucidum* mycelia induced miRNAs regulation in suppressed HepG2 cells

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ABSTRACT

Medicinal mushroom polysaccharides such as *Ganoderma lucidum* polysaccharides (GLPs) have been commonly hypothesized to suppress tumor cells proliferation through immune effects. To verify this hypothesis through investigating comprehensive miRNA expression in polysaccharide treated cancer cells, an anticancer mycelia GLP was employed to disclose miRNA differential expression of human hepatocarcinoma cells (HepG2), by using a miRNA microarray assay based on Sanger miR-Base Release 16. The experiment and the analysis result indicates that among the 61 differential expressed miRNAs ($p \leq 0.01$), 17 of them were regulated significantly. GLP can inhibit HepG2 cells directly through regulation of hepatocarcinoma genes. A newly found miR-3131 exhibited the strongest upregulation (92-folds, Log 2 = 6.53, $p = 0.000016$). The miRNAs responded synergistically in both hepatocarcinoma and immune-related aspects.

1. Introduction

Polysaccharides isolated from *Ganoderma lucidum* (GLPs) have been frequently reported on their in vivo or in vitro anti-cancer activity, as well as the immune-related effect. Based on many results from immune-related cells experiments, a hypothesis has been proposed and widely accepted that GLP probably suppresses tumor genesis or inhibits tumor growth through affecting immune cells and immune-related cells, or, through direct cytotoxic effect and anti-angiogenic effects (Juan, Hsu, Huang, Chen, & Wong, 2011; Xu, Chen, Zhong, Chen, & Wang, 2011; Zheng, Jia, Zhao, Wei, & Liu, 2012). Nevertheless, there has not been a comparative proof to verify the immune-carcinoma relevance, such as a fundamental profile from inhibited cancer cell, for example, the miRNA expression. miRNAs may play two roles in human cancers, either as oncogenes to down-regulate tumor suppressor genes, or as onco-suppressors to target on the molecules involved in promotion of tumor growth. Some hepato-specific miRNAs and their roles have been found and revealed (Monzo et al., 2008; Su et al., 2009), but more is left behind. Hence miRNA expression may provide direct genetic profiles to verify that if the inhibition was immune-related and/or hepatocarcinoma-related.

To date, there is no report about polysaccharide that regulated carcinoma or even immune-related miRNA, except its analogies lipopolysaccharides (LPS). For example, 18 microRNAs were dysregulated 1.5 fold or more in response to lipopolysaccharide, including miR-17-90 cluster (positive to anti-apoptotic functions), and the miR-181 family (positive to T cell tolerance) (Bala et al., 2011). Bacterial LPS induced a reactive oxygen species-/NF-kappa B-dependent pathway, which increased the expression of the anti-inflammatory miR-146a (Schmelzer et al., 2009); that is a connection between the anti-inflammatory and anti-cancer effects, but it still did not disclose both of immune and carcinoma related genetic proof simultaneously.

The fermented intracellar (mycelia) polysaccharides have similar structures and compositions as that of polysaccharides from *G. lucidum* spores or fruit bodies (Petre, Teodorescu, Tuluca, Bejan, & Andronescu, 2010). Previously, we found that the fermented mycelia GLP inhibited the growth of HepG2 cells and induced the G1 phase arrest (Liu, Shen, Xia, Zhang, & Park, 2012). The objective of the current study is to verify the miRNA profile from HepG2 cells treated with the GLP; then based on the cross analysis of reported miRNAs with known functions, to elucidate primary information of the GLP performance in regulation of hepatocarcinoma and immune-related miRNA, using a microfluidic human miRNA array patterned basing on Sanger miR-Base Release 16.

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2. Materials and methods

2.1. Materials and reagents

The human hepatocarcinoma cell lines (HepG2) was provided by the Korea Cell Line Bank (Seoul, Korea). The cells were cultured in DMEM medium containing 10% fetal bovine serum, 1% glutamine (200 mmo1/L), penicillin (100 IU/mL) and streptomycin (100 mg/L) in a humidified 5% CO_2 atmosphere at 37 °C before use. All other reagents were of analysis grade and used directly as received unless stated.

2.2. Preparation of the intracellar GLP

The intracellular GLP were prepared and characterized as described in the supplementary material. The total sugar of the GLP was 93.9% by weight. The molar ratio of the monosaccharides of the GLP is, Ara:Gal:Glc:Xyl:Man = 1.0:2.3:41.0:2.6:2.0. Content of uranic acid in the intracellular GLP is 5.45% by weight. Two main polysaccharides exist in similar ratio in the GLP and possess a molecular weight as 849.5 kDa and 8.0 kDa, respectively.

2.3. Cell culture, miRNA profile and the statistics analysis

The HepG2 cells in logarithmic growth phase were chosen for the experiment as described in the supplementary material.tRNA was extracted using TRK-1001(LC Sciences, USA) for size fractionating to miRNA. An oligonucleotide tag was ligated to the poly (A) tail for later fluorescent dye staining; two different tags were used for the two RNA samples in dual-sample experiments. The microarray assay (μParaflo™ MicroRNA microarray Assay) features, probe modification, tags and hybridization were described in the supplementary material.

Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression). For the two color experiments, the ratio of the two sets of detected signals (Log 2 transformed, balanced) and *p*-values of the *t*-test were calculated; differentially detected signals were those with *p*-values less than 0.01.

2.4. Real-time quantitative RT-PCR verification for mature miRNA

To quantify expression of the mature miRNA, total miRNA was extracted using miRNeasy mini kit (Qiagen, Valencia, CA, USA). Real-time PCR for monitoring the expression of mature miRNA was carried out using miScript PCR Starter Kit and miScript Primer Assay specific for the mature miRNA (catalog number: MS00020538, Qiagen, Valencia, CA, USA). The real-time PCR was performed on a CFX96 real-time PCR system (Bio-Rad) with QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA, USA). In quantifications, RNU6B (RNU6-2) was assessed as the internal control, and data were normalized using the 2-ΔΔCT method. Detailed futures are described in the supporting material.

3. Results and discussion

3.1. miRNA expression of the HepG2 cells exposed to the intracellar polysaccharide

To obtain comprehensive information of miRNA differential expression, human miRNA-based array layout was chosen and designed to verify the miRNA differential expression of HepG2 cells

Table 1
miRNA regulation of the GLP on *HepG2* cells.

Reporter name[a]	*p*-Value	Log 2-value[b]	Reporter name[a]	*p*-Value	Log 2-value[b]
hsa-miR-3131	1.59E–05	6.53	hsa-miR-25	1.29E–04	–0.54
hsa-miR-572	1.05E–03	2.54	hsa-miR-26a	7.62E–03	–0.66
hsa-miR-4281	1.08E–03	2.22	hsa-miR-107	3.90E–03	–0.77
hsa-miR-1246	5.26E–04	1.66	hsa-miR-92a	1.56E–03	–0.78
hsa-miR-762	3.87E–04	1.34	hsa-miR-20a	8.02E–03	–1.03
hsa-miR-574-5p	4.20E–03	1.30	hsa-miR-92b	5.16E–03	–1.16
hsa-miR-3141	3.29E–03	1.01	hsa-miR-23a	1.15E–03	–1.45
hsa-miR-663	3.90E–03	0.95	hsa-miR-10b	2.62E–04	–1.77
hsa-miR-2861	7.83E–03	0.68			
Reporter Name[c]	*p*–value	Log2–value[b]	Reporter name[c]	*p*–value	Log2–value[b]
hsa-miR-1224-5p*	4.67E–03	2.39	hsa-miR-484	2.58E–03	–0.44
hsa-miR-3149	1.70E–04	2.35	hsa-miR-345	9.98E–04	–0.54
hsa-miR-483-5p	1.51E–03	1.87	hsa-miR-30a	2.06E–03	–0.74
hsa-miR-939	3.13E–03	1.77	hsa-miR-454	7.16E–03	–0.79
hsa-miR-671-5p	4.21E–04	1.74	hsa-miR-34c-3p	8.82E–04	–0.91
hsa-miR-4270	2.84E–04	1.74	hsa-miR-151-3p	3.50E–03	–0.91
hsa-miR-1208	2.18E–03	1.71	hsa-miR-103-2*	4.89E–03	–0.99
hsa-miR-188-5p	2.74E–03	1.70	hsa-miR-148b	1.87E–03	–1.02
hsa-miR-601	8.83E–04	1.52	hsa-miR-19b	3.05E–03	–1.04
hsa-miR-494	4.15E–03	1.44	hsa-miR-505*	4.54E–03	–1.08
hsa-miR-3610	5.33E–03	1.42	hsa-miR-27a	1.08E–03	–1.16
hsa-miR-1228*	5.32E–03	1.42	hsa-miR-15a	8.47E–03	–1.24
hsa-miR-1268	1.14E–03	1.40	hsa-miR-3613-3p	6.21E–03	–1.45
hsa-miR-877*	3.12E–03	1.33	hsa-miR-3663-3p	3.12E–03	–1.48
hsa-miR-513a-5p	2.76E–03	1.15	hsa-miR-3182	3.30E–03	–1.52
hsa-miR-1973	1.01E–03	1.15	hsa-miR-23c	1.24E–03	–1.72
hsa-miR-568	2.63E–03	1.06	hsa-miR-497	4.21E–03	–2.08
hsa- miR-489	3.56E–03	1.03	hsa-miR-199a-3p	5.38E–03	–2.20
hsa-miR-134	1.84E–03	0.96	hsa-miR-196b*	4.67E–04	–2.51
hsa-miR-765	8.44E–03	0.93	hsa-miR-605	8.01E–03	–2.73
hsa-miR-3178	1.65E–03	0.90			
hsa-miR-1260	4.79E–03	0.86			
hsa-miR-3195	4.17E–03	0.85			
hsa-miR-1238	8.55E–03	0.83			

[a] transcripts having strong signals (signal ≥ 500).
[b] sample/control.
[c] transcripts having low signals (signal < 500).

Table 2
Hepatic miRNAs possessed significant differential expression.

miRNA	Source	Regulation	Drug	Reference and function suggested if any
miR-217 miR-518b miR-517c miR-520g miR-519a miR-522 miR-518e miR-525-3p miR-512-3p miR-518a-3p	Human HCC and adjacent non-tumor tissues; inhibit HepG2 and Huh7	Upregulated	None	(Wang, Zhao, Tan, Ren, & Qi, 2012)
miR-138 miR-214 miR-214# miR-27a# miR-199a-5p miR-433 miR-511 miR-592 miR-483-5p miR-483-3p		Downregulated	Mimic or inhibitor of miR-138	miR-138 can regulate CCND3 as a tumor suppressor in HCC, and inhibit HepG2 and Huh7 proliferation by inducing cell cycle arrest at G1/S phase (Wang, Zhao, Tan, Ren, & Qi, 2012)
miR-27b miR-181a miR-146b-5p miR-181d miR-146a miR-15a/16-1 Cluster	Huh-7 HepG2	Upregulated N/A	Adramycin, cisplatin, carboplatin, mitomycin C, vincristine None	(Zhuo, Liu, Wang, Gao, & Huang, 2013) Regulating PCMT1 (Sambri, Capasso, Pucci, Perna, & Ingrosso, 2011)
miR-21 miR-122 miR-223	Serum of patients with HCC	Upregulated	None	Xu et al., 2011
miR-34a	Liver tissue in Muta (TM) Mouse	Upregulated	Benzo(a)pyrene	Involved in p53 response (Malik, Williams, Lemieux, White, & Yauk, 2012)
miR-101	HCC tissues, HepG2, Hep3B, SK-Hep1, Huh7, QGY-7703, and SMMC-7721	Downregulated	None	Targeting Mcl-1 (Su et al., 2009)
miR-145	Cancer stem-like cells (T3A-A3) derived from hepatocarcinoma	Downregulated	None	Potentially via modulation of the downstream target, (Jia et al., 2012b)
miR-155	Kupffer cells from livers of female mice (C57BL/6)	Upregulated	Gut-derived lipopolysaccharide (LPS) and Toll-Like Receptors 4 (TLR4)-LPS	Regulate production of TNF (Bala et al., 2011)
miR-192	HepG2.2.15 cells	Upregulated	None	Inhibit nucleotide excision repair by targeting ERCC3 and ERCC4 (Xie et al., 2011)
miR-199a*		Downregulated in cancer cells, upregulated by lentivirus	Lentivirus	Partly through downregulation of HIF-1α (Jia et al., 2012a)
miR-203 miR-124	19 liver cancer cell lines (cHc4, Hep 3B, Hep G2, Hep-KANO, Hep-TABATA, HLE, HLF, huH-1, HUH-6, Huh7, JHH-1, JHH-2, JHH-4, JHH-5, JHH-6, JHH-7, Li7, PLC/PRF/5 and sK-Hep-1)	Inversely correlated with methylation status	5-aza-2′-deoxycytidine	(Furuta, Kozaki, Tanaka, Arii, Imoto, & Inazawa, 2010)
miR-221 miR-222 miR-21 miR-31 miR-122	HCC tissues	Upregulated	None	(Karakatsanis, Papaconstantinou, Gazouli, Lyberopoulou, Polymeneas, & Voros, 2013)
miR-145 miR-146a miR-200c miR-223	HCC tissues	Downregulated	None	(Karakatsanis, Papaconstantinou, Gazouli, Lyberopoulou, Polymeneas, & Voros, 2013)
miR-122	HepG2 QGY-7703	Not applicable	None	Regulates cholesterol metabolism and promotes hepatitis C virus (HCV) replication (Hsu et al., 2012; Narbus et al., 2011; Negrini, Gramantieri, Sabbioni, & Croce, 2011)

Table 2 (*Continued*)

miRNA	Source	Regulation	Drug	Reference and function suggested if any
miR-373	HCC tissues	Upregulated	None	Regulator of protein phosphatase 6 (Wu et al., 2011)
miR-450a	HCC tissues	Downregulated	None	Inhibite DNMT3a expression (Weng et al., 2011)
miR-503	HCCLM3 MHCC97-L	Downregulated	None	Induce a G1 arrest and decrease proliferation for HCCLM3 cell (Zhou & Wang, 2011)

*Transcripts having strong signals (signal≥500).

Table 3
Hepatic miRNAs with significant differential expression in HepG2 induced by the GLP.

Hepatic miRNA	Regulation	[a]Regulation reported in literature	miRNA source in literature	Reference and function suggested if any
miR-10b	Downregulated	Upregulated	HepG2 cells	Increased cell migration and invasion (AAi, Wu, Wen, Xu, Lv, & Wu, 2011)
miR-23a	Downregulated	Upregulated	Primary human HCC	(Wang, Hsu, Frankel, Ghoshal, & Jacob, 2012)
miR-92a	Downregulated	Upregulated	HCC	Shigoka et al. (2010)
miR-199a-3p	Downregulated	Upregulated	In human fibrotic liver tissue	Lee et al. (2012)

[a] Regulation reported in cancer tissue/cell comparing to normal tissue/cell from literature.

treated with GLP (Table 1). More attention was paid to the differential expression of hepatocarcinoma and immune-related miRNAs in the analysis thereafter.

As presented in Table 1, 17 reporters presented strong response (signal reading >500, $p \leq 0.01$) and significant regulation, as downregulated miRNAs (miR-25, miR-26a, miR-107, miR-92a, miR-20a, miR-92b, miR-23a and miR-10b) and upregulated miRNAs (miR-572, miR-4281, miR-1246, miR-762, miR-574-5p, miR-3141, miR-663 and miR-2861). In addition, there are many newly found miRNAs in Table 1 that have been seldom reported about their function.

Among the 61 responded miRNAs, miR-3131 was upregulated 92-folds (Log 2 = 6.53, $p = 0.000016$). miR-3131 has been barely reported and its function has not been revealed in any literature; it was only reported in upregulation (Log 2 = 1.14) in six malignant cell lines irradiated with 6 MeV photons (Niemoeller et al., 2011).

Because of the short history and fast growing database, hepatic including hepatocarcinoma microRNA have not been reviewed in detail Lendvai et al. (2012). Apart from miRNAs presented in Table 1, other hepatocarcinoma miRNAs reported in literatures are summarized in Table 2 for the purpose of comparison and discussion.miRNAs shown in Table 1 consist of hepatic and non-hepatic microRNAs, some of them are known to be functional or multi-functional; while roles of the others have not been clarified. The well-known hepatic miRNAs in Table 1 are miR-10b, miR-23a, miR-92a, and miR-199a-3p, all of their regulation devoted a beneficial effect on the cell suppressing as shown in Table 3. This result clearly indicated that the GLP can inhibit HepG2 cells directly through regulation of hepatocarcinoma genes. To date, such direct proof has not been reported.

As mentioned previously, many miRNAs respond to immune or immune-related cells (Bala et al., 2011). As expected, some immune-related or other cancer related miRNAs are presented in Table 1. The beneficial regulations including miR-1246 (Log 2 = 1.66), which has been recently claimed as a novel target of p53, p63 and p73 to suppress the expression and consequently activate NF-AT (T cells transcription factor), both of which are involved in down syndrome and possibly with tumourigenesis (Zhang, Liao, Zeng, & Lu, 2011). GLP was also found to induce macrophage-like differentiation in human leukemia THP-1 cells via caspase and p53 activation (Juan et al., 2011). As for miR-34a, it was the only responded miRNA in a global miRNA expression in Muta (TM) mouse exposured to benzo(a)pyrene (3.6-fold increase, adjusted $p < 0.05$), which is involved in p53 response (Malik, Williams, Lemieux, White, & Yauk, 2012). miR-92b has been commonly found in cancers; inhibition of miR-92b (Log 2 = −1.16) in human ES cells resulted in a greater number of cells in the G1 phase and a lower number in the S phase (Sengupta, Nie, Wagner, Yang, Stewart, & Thomson, 2009), however miR-92b also suppressed epithelial di/tripeptide membrane transporter PepT1(Dalmasso et al., 2011).

Other non-hepatic miRNAs with beneficial regulation includes miR-3663-3p (Log 2 = −1.48, upregulated in cutaneous malignant melanoma (Sand et al., 2013)); miR-199a-3p (Log 2 = −2.2, a marker over-expressed in gastric cancer (Li et al., 2012), miR-20a (Log 2 = −1.03, rich in chronic lymphocytic leukemia cells (Zhu et al., 2012), miR-663 (Log 2 = 0.95, less expression in human gastric cancer cells (Pan et al., 2010) and helping trans-retinoic acid to differentiate the acute myeloid leukemia cell line HL-60 (Jian et al., 2011)).

Nevertheless, not all the miRNA regulations are beneficial, such as the 4 upregulated non-hepatic miRNAs including miR-4281 (upregulated in cutaneous malignant melanoma (Sand et al., 2013)), miR574-5p (increasing the risk of esophageal cancer (Yang et al., 2013)); while miR-572 is one of the most frequent downregulated miRNAs in chronic lymphocytic leukemia cells (Zhu et al., 2012) but presents high level in liver injury resulted from chronic hepatitis B and multiple sclerosis (Siegel, Mackenzie, Chaplin, Jablonski, & Griffiths, 2012). miR-199a-3p has also been reported to be either upregulated or down-regulated in a variety of tumors (Sakurai et al., 2011).

The intracellular GLP has been confirmed can suppress cell proliferation; the experiment results in current study indicate that it is associated with miRNAs differential expression. Except immune-related and hepatocarcinoma-related effect, in the hepatocarcinoma inhibition pathway of GLP, there could be many other connections between various bioeffects (for example, antioxidation) and anticancer effects; which will need numerous works on database construction. This will update the findings of polysaccharides' performance in miRNA regulation, and could expand the common hypothesis that GLP from *G. Lucidum* suppresses the tumor growth indirectly.

3.2. Quantitative RT-PCR verification for miR-3131

As shown in Fig. 1, miR-3131 was confirmed to be over-expressed in Hep2 cells after treatment with GLPs. Quantitative RT-PCR results validated the microarray results which was 4.78

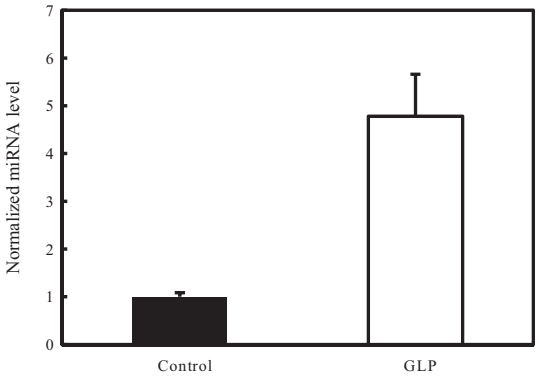

Fig. 1. Quantitative RT-PCR verification for mature miRNA.

times over-expressed in the GLP treatment group; $p = 0.0096$ (additional information is presented in the supporting material).

4. Conclusions

The experiment results clearly revealed that the GLP can suppress Hep2 cells via the regulation of the hepatic miRNAs and immune-related miRNAs. Among the 61 regulated miRNA, miR-3131 represented the strongest upregulation ($\text{Log}\,2 = 6.53$, $p = 0.000016$). The regulation of hepatic miRNAs (miR-10b, miR-23a, miR-92a, and miR-199a-3p) and non-hepatic miRNAs (miR-1246, miR-20a, and miR-92b) devoted significant positive effect to the inhibition of GLP's on HepG2, while non-hepatic miR-4281, miR574-5p contributed unbeneficial effects to the cell suppressing. In addition to miR-3131, many 4 digital code miRNAs were found to be involved in this polysaccharide induced differential expression, as long as their targets could be found, their function and subsequently the functions of polysaccharides in cancer treatment will be revealed gradually.

Conflicts of interests

No conflicts of interests were declared by the authors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.12.044.

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