# Analyses of the possible anti-tumor effect of Yokukansan

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**Abstract**

The Kampo medicine Yokukansan (YKS) has a wide variety of properties such as anxiolytic, anti-inflammatory and analgesic effects, and is also thought to regulate tumor suppression. In this study, we investigated the anti-tumor effect of YKS. We used Lewis lung carcinoma (LLC)-bearing mice that were fed food pellets containing YKS and then performed a fecal microbiota analysis, a microarray analysis for microRNAs (miRNAs) and an *in vitro* anti-tumor assay. The fecal microbiota analysis revealed that treatment with YKS partly reversed changes in the microbiota composition due to LLC implantation. Furthermore, a miRNA array analysis using blood serum showed that treatment with YKS restored the levels of miR-133a-3p/133b-3p, miR-1a-3p and miR-342-3p following LLC implantation to normal levels. A TargetScan analysis revealed that the epidermal growth factor receptor 1 (EGFR1) signaling pathway is one of the major target pathways for these miRNAs. Furthermore, treatment with YKS restored the levels of miR-200b-3p and miR-200c-3p, a recognized mediator of cancer progression and controller of emotion, in the hypothalamus of mice bearing LLC. An *in vitro* assay revealed that a mixture of pachymic acid, saikosaponins a and d and isoliquiritigenin, which are all contained in YKS, exerted direct and additive anti-tumor effects. The present findings constitute novel evidence that YKS may exert an anti-tumor effect by reversing changes in the fecal microbiota and miRNAs circulating in the blood serum and hypothalamus, and the compounds found in YKS could have direct and additive anti-tumor effects.

**Keywords:** yokukansan, tumor, microRNA, microbiota, hypothalamus

**Abbreviations**

CF, Control food; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; DNMT3a, DNA methyltransferase 3a; EGFR, epidermal growth factor receptor; EMT, epithelial-to-mesenchymal transition; GM, geissoschizine methyl ether; HPA, hypothalamic-pituitary-adrenal; IL-6, Interleukin-6; LLC, Lewis lung carcinoma; miRNAs, microRNAs; N.Acc, nucleus accumbens; NO, nitric oxide; NSCLC, non-small cell lung cancer; NT, non-tumor; STAT, Signal Transducers and Activators of Transcription; TLR, Toll-like receptor; YKS, Yokukansan; YKSF, YKS-treated food; Zeb2, Zinc finger E-box-binding homeobox 2.

**Introduction**

Psychological stress, such as that associated with depression or social isolation, has been identified as an important factor in tumor growth [1]. Psychosocial stress affects sympathetic, neuroendocrine, and immune responses, resulting in facilitation of the hypothalamic-pituitary-adrenal (HPA)-axis and sympathetic nervous system, and subsequently contributes to the progression of tumor growth through the regulation of inflammatory responses by immune cells [2].

MicroRNAs (miRNAs) are small nucleotide sequences of non-coding RNA that regulate the expression of transcriptome involved in embryonic development, differentiation, proliferation, and apoptosis [3]. In the field of pain research, we previously reported that the expression of miR-200/429 was dramatically decreased in the nucleus accumbens (N.Acc), which is the terminus of the reward system, by neuropathic pain-like stimuli [4]. We also found that sciatic nerve ligation changed the expression of many miRNAs in the amygdala, which is the main brain region for

controlling negative emotion [5]. Furthermore, it has recently been reported that the miR-200 family is related to several malignancies, including lung cancer, breast cancer, ovarian cancer, prostate cancer, lymphoma, pancreatic cancer, colorectal cancer and gastric cancer [6]. The miR-200 family helps to regulate the epithelial-to-mesenchymal transition (EMT), cancer cell proliferation, and drug resistance [7].

The gut microbiota has a varied and multifactorial cell density and composition. Shifts in the composition and diversity of the gut microbiota have been associated with carcinogenesis [8], tumor immunotherapy [9], and anticancer immunosurveillance [10], as well as interactions with the host immune system and cancer development and treatment [11].

Yokukansan (YKS) is a Kampo medicine consisting of seven medicinal herbs, including *Uncaria* hook, *Bupleurum* root, Japanese *Angelica* root, *Cnidium* rhizome, *Atractylodes lancea* rhizome, *Poria* sclerotium, and *Glycyrrhiza* root [12]. Recent basic studies have addressed the efficacy of YKS for treating psychological stress and anxiety via the activation of serotonin 5-HT$_{1A}$ receptors [13], a cholinergic mechanism [14], modification of glutamatergic neuron activity [15] and regulation of orexin secretion [16], along with anti-inflammatory [17] and analgesic effects [18]. Since YKS has multiple actions associated with its anti-stress and anxiolytic effects, it can also be expected to regulate tumor suppression. However, little is known about tumor suppression by YKS or its mechanism.

In this study, we clarified the effect of YKS on tumor growth. Furthermore, we investigated changes in the composition of the gut microbiota and the expression of circulating miRNAs, and also performed cell viability assays on some of its component compounds that can be expected to have an anti-tumor effect.

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**Materials and Methods**

**Animals**

  This study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. We performed the experiments using male C57BL/6J mice (Tokyo Laboratory Animals Science, Tokyo, Japan), aged 7 weeks at the start of the experimental procedures. After the implantation of LLC cells, animals were individually housed in a room with an ambient temperature of $23 \pm 1$ °C and a 12 hr light-dark cycle (lights on 8:00 a.m. to 8:00 p.m.).

**Drugs and administration**

  Yokukansan consisted of seven medicinal herbs: 4.0 g of *Atractylodes lancea* rhizome, 4.0 g of *Poria* sclerotium, 3.0 g of *Cnidium* rhizome, 3.0 g of *Uncaria* hook, 3.0 g of Japanese *Angelica* root, 2.0 g of *Bupleurum* root, and 1.5 g of *Glycyrrhiza* root. It was supplied by Tsumura & Co. (Tokyo, Japan) as a powdered mixture of dried herbal extracts. Control food (CF) was standard pellet chow for rodents (Oriental Yeast Co., Ltd.). For YKS-treated food (YKSF), powdered Yokukansan was mixed into CF at a concentration of 0.6%. We measured the food intake and body weight of each mouse daily. Food and water were freely available during the experiment.

**Graft tumor growth assay**

We anesthetized the animals with isoflurane (3%; Wako Pure Chemical Industries, Tokyo, Japan). LLC cells, which belong to *Kras*-mutant cell line [19], were resuspended in a mixture of extracellular matrix (ECM) gel (Sigma-Aldrich Co., St. Louis, MO) and Hank's Balanced Salt Solution (HBSS, Thermo Fisher Scientific Inc., Waltham, MA) (3:1) at a concentration of $2 \times 10^6$ cells/0.5mL, and 0.5mL of this suspension was then transplanted subcutaneously into the right lower back of mice. Tumor volume was measured at 4, 7, 11, 14, 18, and 21 days using a caliper, and calculated as $(L \times W^2)/2$, (L: length and W: width).

**Microarray analysis for microRNAs**

Total RNA, including miRNAs, was extracted from the serum of 1 mouse per group at 22 days after LLC cell implantation, using a mirVana PARIS Kit (Thermo Fisher Scientific). Agilent Mouse 8×60K expression Microarrays were used to profile miRNA expression. All of the procedures for microarray analysis were performed by DNA Chip Research Inc. (Tokyo, Japan).

**miRNA target prediction and pathway analysis**

To predict miRNA target mRNAs, TargetScan Mouse 7.1 (www.targetscan.org) was used. The resulting target mRNA lists for each miRNA were integrated using a Venn diagram with GeneSpring 14.5 (Agilent Technologies). The integrated target mRNA list was subjected to a pathway analysis through a Simple Experiment Analysis using the WikiPathways database.

**Quantification of miRNAs with real-time PCR (qRT-PCR)**

Mice were sacrificed by decapitation at 22 days of treatment with YKS. Total RNA extracted from blood serum and brains of mice were isolated for the RT reaction and transcribed cDNA was then used for subsequent PCR amplification with Universal PCR Master Mix, No AmpErase UNG (Thermo Fisher Scientific). Real-time PCR was conducted at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The threshold cycle (Ct) method was used to determine the relative quantity of each miRNA.

**Fecal microbiota sequencing**

Fecal boli of each mouse were immediately collected, frozen in dry ice, and then stored at -80°C until extraction. For 16S ribosomal RNA (rRNA) sequencing, the V3 to V4 region of 16S rRNA genes from extracted DNA were amplified using bacterial universal primers. The purified amplicons (average 500-bp) were pooled in equimolar amounts and used as a template to prepare a sequencing library. Sequencing was performed using an Illumina MiSeq (Illumina, San Diego, CA). Data processing, assignment, and data aggregation were performed using Repertoire analysis software (Repertoire Genesis, Tokyo, Japan). Cluster analysis was performed with GeneSpring software (Agilent Technologies, CA, USA).

**Cell culture**

The LLC cell line was cultured in RPMI 1640 medium HELPES Modification (Sigma-Aldrich) with 10% fetal bovine serum (FBS; Invitrogen™ Life Technologies

Co., Carlsbad, CA) and penicillin-streptomycin (PS; Invitrogen™). Cell culture was maintained in a water-humidified 37°C incubator with 5% $CO_2$.

**Reagents**

The reagents used in this study consisted of seven compounds contained in YKS: pachymic acid, isoliquiritigenin, saikosaponin a (Toronto Research Chemicals Inc., Toronto, Canada), saikosaponin c, saikosaponin d, β-eudesmol (FUJIFILM Wako Pure Co., Osaka, Japan), and palmitic acid (Combi-Blocks Inc., San Diego, CA).

**MTT assay**

LLC cells were seeded at a density of $5 \times 10^4$ cells/ml. Seven compounds at concentrations of 1, 3, and 10 μM and a 4-compound mixture at concentrations of 1 and 3 μM were added to the cells, which were then incubated for 48 hr. After incubation, 20 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl) 22,5-diphenyltetrazolium bromide (MTT) was added to each well for 2 hr, and then the reaction was stopped by replacing the media with 100 μL of DMSO. Optical density analysis was performed using a microplate reader at an absorption wavelength 600 nm.

**Statistical analysis**

Data are expressed as means ± SEM. Differences between groups were analyzed for significance using the unpaired Student's *t*-test or one-way ANOVA with Tukey's *post hoc* test. A value of $p < 0.05$ was considered statistically significant. Data involving time-dependent changes were analyzed using two-way repeated measures (RM)

ANOVA followed by Bonferroni *post-hoc* tests, where appropriate. All statistical analyses were performed with Prism version 5.0 (GraphPad Software, La Jolla, CA).

## Results

### Effect of YKS on tumor development

To investigate whether YKS affects tumor growth, we used LLC-bearing mice fed control food (CF) pellets containing 0.6% YKS (YKSF; about 0.9 g/kg/day) and measured their tumor volumes. Treatment with YKSF was started after LLC implantation and continued for 3 weeks (Fig. 1A). While tumor growth proceeded gradually after tumor implantation in all mice in the LLC-implanted groups, the tumor volume in the YKSF-LLC group was less than that in the CF-LLC group. The difference between the CF-LLC and YKSF-LLC groups was statistically significant at 21 days after LLC implantation ([***]$p<0.001$, Fig. 1B). During the experimental period, there were no significant differences in food intake or changes in body weight between the two groups (Fig. 1C, D). These results suggest that treatment with YKS for 3 weeks may suppress tumor growth.

### Regulation of the gut microbiota composition by YKS treatment

Recent studies have suggested that there could be an association between the gut microbiota and antitumor immunity [20]. Therefore, we examined whether YKSF feeding could affect the composition of the gut microbiota. Unexpectedly, the overall gut microbiota compositions were not different among the non-tumor and control food (CF-NT) group, tumor-bearing and control food (CF-LLC) group, non-tumor and control food containing 0.6%YKS (YKSF-NT) group, and tumor-bearing and control

food containing 0.6% YKS (YKSF-LLC) group (Fig. 2A). Next, we analyzed the gut microbiota at the species level and found that the CF-LLC group had significantly lower levels of *Bacteroidales, Desulfovibrionaceae* and *Veillonellaceae* than the CF-NT group ($^*p<0.05$, $^{**}p<0.01$, Fig. 2b). Furthermore, the reductions in *Bacteroidales* and *Desulfovibrionaceae* in the YKSF-LLC group were significantly less than those in the CF-LLC group ($^{\#}p<0.05$, Fig. 2B) and a similar though non-significant trend was noted for *Veillonellaceae*. These data suggest that the gut microbiota composition changed under the tumor-bearing condition, and that these changes could be reduced, at least in part, by treatment with YKS.

**Changes in miRNA expression under YKS treatment**

To evaluate whether YKS could affect miRNA expression levels in blood serum, a miRNA array analysis was performed. Scatterplots comparing the expression profiles of CF-NT and CF-LLC, or CF-NT and YKSF-LLC are shown in Fig. 3A and 3B, respectively. The expression of many miRNAs was altered in the LLC-bearing condition. While specific miRNAs were up- or down-regulated (> 2-fold change) in the CF-LLC group, some of these changes were reversed to the respective normal levels in the YKSF-LLC group. Among them, miR-133a-3p/133b-3p and miR-1a-3p were found to be clearly up-regulated in the CF-LLC group, and these changes were restored by YKS treatment. In contrast, miR-342-3p was down-regulated in the CF-LLC group, and this effect was slightly reduced by YKS (Fig. 3C). We quantitatively validated the changes in miR-133a-3p, miR-133b-3p, miR-1a-3p and miR-342-3p using a qRT-PCR assay (Table 1).

**Prediction of mRNA targets for miR-133a-3p/133b-3p, miR-1a-3p, and miR-342-3p**

To identify the candidate mRNA targets for miR-133a-3p/133b-3p, miR-1a-3p and miR-342-3p, we used Target Scan Mouse 7.1. Target mRNA lists for each miRNA were integrated using a Venn diagram with GeneSpring 14.5. We obtained a total of 1475 target genes (Fig. 3D). We extracted the top 10 pathways from a WikiPathway analysis with all of the target mRNA candidates, and found that the EGFR1 signaling pathway was included in each of the top 10 pathways (Fig. 3E). Indeed, 26 target mRNAs were enriched in the EGFR1 signaling pathway (Fig. 3F). These results suggest that YKS may exert an anti-tumor effect by modulating the EGFR1 signaling pathway.

**miRNA expression levels in the hypothalamus**

To examine the effects of YKS on the brain, we performed a quantitative analysis of miRNA expression in the hypothalamus using qRT-PCR. Expression levels of members of the miR-200 family such as miR-200b-3p and miR-200c-3p, which would play a critical role in cancer progression, brain dysregulation and emotionality, were significantly down-regulated in the CF-LLC group compared to the CF-NT group ($^{**}p<0.01$, $^{***}p<0.001$, Fig. 4A, B). Interestingly, these reductions were significantly reduced in the YKSF-LLC group compared to the CF-LLC group ($^{#}p<0.05$, Fig. 4A, B). The expression level of another miR-200 family member, miR-429-3p, was significantly decreased by LLC (Fig. 4C). This decrease in the level of miR-429-3p in the YKSF-LLC group was slightly reduced compared to that in the CF-LLC group (Fig. 4C). The expression levels of another emotion-controller, miR-34c-5p, but not miR-34b-3p, were significantly increased in both the YKSF-NT and CF-LLC groups

($^{**}p$<0.01, Fig. 4D, E). However, there were no significant differences in the expression of miR-34b-3p or miR-34c-5p between the CF-LLC and YKSF-LLC groups (Fig. 4D, E).

**Cell viability by the MTT assay**

 While pachymic acid, saikosaponins a and d, and isoliquiritigenin significantly decreased cell viability at 10 µM, saikosaponin c, palmitic acid, and β-eudesmol did not influence cell viability (Fig. 5A). Further MTT assays were performed in the presence of pachymic acid, saikosaponins a and d, and isoliquiritigenin, both separately and as a 4-component mixture. Pachymic acid, saikosaponin a and isoliquiritigenin significantly decreased cell viability in a concentration-dependent manner at 1 and 3 µM. In the same concentration 4-compound mixture, an additive decrease in cell viability was clearly observed compared to pachymic acid alone (Fig. 5B).

**Discussion and conclusions**

 It has been reported that YKS has anti-inflammatory, anti-stress and analgesic effects, and YKS is commonly used to treat neurosis, insomnia and dementia [21-23], and these symptoms are often seen in cancer patients [24]. In the present study, we found that oral intake of YKS for 3 weeks suppressed tumor growth in LLC-bearing mice. These results suggest that YKS may be effective for tumor suppression.

 Recently, the gut microbiota has been considered to be associated with many diseases, including cancer. Therefore, we investigated whether the composition of the gut microbiota could be changed by YKS treatment. As a result, *Bacteroidales, Veillonellaceae* and *Desulfovibrionaceae* were reduced in the CF-LLC group, and this

effect was itself reduced in the YKSF-LLC group. Interestingly, it has recently been reported that *Bacteroidales* has an anti-tumor effect via cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) [25]. Although further investigation is required, the effect of YKS may involve, at least in part, CTLA-4 regulation. Although the mechanism of YKS-induced recovery from the decrease in gut microbiota in the process of tumor growth has not been identified yet, the previous report showed that isoliquiritigenin, which is a component compound of YKS, could improve the gut microenvironment under the state of cancer [26]. Therefore, we hypothesize here that this effect may, at least in part, contribute to the suppression of tumor progression.

Another key finding of the present study was that YKS treatment reduced changes in the expression levels of circulating miRNAs in blood serum and brain-related miRNAs in the hypothalamus of tumor-bearing mice. Among miRNAs that were altered in blood serum, the effects on miR-133a-3p/133b-3p, miR-1a-3p and miR-342-3p were reduced by YKS treatment. Thus, we sought to identify potential common targets of miR-133a-3p/133b-3p, miR-1a-3p and miR-342-3p, and found that one of these common targets was the EGFR1 signaling pathway. EGFR has been shown to be implicated in carcinogenic processes such as cell survival, proliferation, metastasis and angiogenesis [27]. This type of cancer EGFR is considered to be one of the most potent drivers of tumor development, and is a critical target for lung cancer therapies [28]. In fact, EGFR mutations have been observed in a certain percentage of lung cancers [29]. A growing body of evidence suggests that EGFR is mainly overexpressed in non-small cell lung cancer (NSCLC) and such overexpression of EGFR is associated with reduced survival, frequent lymph node metastasis, poor prognosis and chemosensitivity [30, 31]. When several specific ligands bind to EGFR, activation of its signaling pathways,

including the RAS/RAF/ERK/MAPK pathway, results in cell proliferation [32]. The PI3K/Akt pathway, and Signal Transducers and Activators of Transcription (STAT) 3 and 5 signal transduction pathways activated by the stimulation of EGFRs are involved in the evasion of apoptosis [33]. Considering this background, we hypothesized that YKS may suppress the tumor-activated EGFR1 signaling pathway while also modulating the expression of blood serum-oriented miRNAs, and contribute to the suppression of tumor growth.

The HPA axis is known to be a pivotal component of an organism's response to stress. Activation of the stress network through the HPA axis may directly interact with immune cell responses [34]. Therefore, we next investigated the functional changes related to miRNAs in the hypothalamus of tumor-bearing mice, which is the brain region that efferent molecules are easy to reach due to the possible damage of the blood-brain barrier in mice with tumor. It has been reported that many miRNAs are expressed and enriched in the hypothalamus, and these miRNAs play important roles in regulating hypothalamic control functions [35]. miR-200b-3p and miR-200c-3p have been considered to be correlated with tumor progression and brain dysfunction such as major depressive disorder, Huntington's disease and cerebral ischemia [36-38]. In the present study, we found that the levels of miR-200b-3p and miR-200c-3p in the hypothalamus, which were both significantly decreased in the CF-LLC group, were significantly restored in the hypothalamus of the YKSF-LLC group. In contrast, no significant differences in miR-34b-3p or miR-34c-5p, both of which are enriched in the brain and critical for control of negative emotions [39, 40], were found between the CF-LLC and YKSF-LLC groups. It has recently been reported that the miR-200 expressed in microglia regulates the microglial inflammatory process and neuronal

survival by modulating the c-Jun/MAPK pathway [41]. Furthermore, the miR-200 family has been shown to control postnatal forebrain neurogenesis via Zinc finger E-box-binding homeobox 2 (Zeb2) inhibition [42]. More importantly, we previously reported that the expression of DNA methyltransferase 3a (DNMT3a) was significantly increased with decrease in miR-200b/429 clusters in the N.Acc. under a neuropathic pain-like state [4]. Taken together, these results suggest that down-regulation of the miR-200 family in the hypothalamus could contribute to brain dysregulation associated with neuroinflammation. Although the direct mechanism of YKS-induced recovery from the decrease in the expression of miR-200s in the hypothalamus in the process of tumor growth should be investigated in future approach, we hypothesize here that YKS may have therapeutic potential for cancer by ameliorating brain dysregulation consequent to changes in the miRNA profile, including the miR-200 family, due to cancer.

  Since YKS is composed of seven medicinal herbs, the overall effect of YKS may result from the synergistic actions of these herbal medicines. Some of these herbs contained in YKS are known to possess anti-tumor activity. Pachymic acid, a lanostane-type triterpenoid contained in *Poria* sclerotium, exerts anti-tumor effects. It can inhibit the growth of several human cancer cells through apoptosis by reducing prostaglandin synthesis and AKT activity [43, 44], activation of the endoplasmic reticulum (ER) stress pathway [45, 46], and the inhibition of mitochondrial capacity [47]. Saikosaponin contained in *Bupleurum* root includes multiple saponins (a,b,c,d,e,f), and the main saponins that are responsible for the anti-tumor effect are considered to be saikosaponins a and d. Saikosaponin a induces apoptosis and cell cycle arrest via the sequential activation of caspase family through the involvement of the p53/p21 pathway

[48-52] and the suppression of phosphatidylinositol 3kinase/Akt and MAPK signaling [53] in several cancer cell lines. Isoliquiritigenin, a natural flavonoid contained in *Glycyrrhiza* root, inhibits the proliferation of human NSCLC A549 cells though the p53 and Fas/FasL apoptotic system [54] and induces cell cycle arrest and p21(CIP1/WAF1) expression [55]. In addition, isoliquiritigenin induced apoptosis and suppressed tumor growth in human NSCLC cells [56]. However, the anti-tumor effects of YKS, which contains these compounds, have not been clarified. In this study, we examined the anti-tumor effects of YKS using a LLC cell xenograft mouse model and further identified the active compounds by MTT assay. Next, we confirmed the anti-tumor effects of pachymic acid, saikosaponins a and d, and isoliquiritigenin, and showed that a mixture of these 4 compounds had an additive anti-tumor effect. Although these components are not included with the same amount in YKS, our findings suggest that YKS contains many compounds with various anti-tumor effects and these exert an additive or a synergistic effect on tumor growth.

In conclusion, our findings suggest that YKS suppresses tumor growth and restores the expression of miR-200b/c-3p in the hypothalamus and miR-133a/b-3p, miR-1a-3p, and miR-342-3p in serum. Furthermore, disruption of the fecal microbiota composition in tumor-bearing mice was partly restored to the control composition by YKS. These results suggest that YKS may exert an anti-tumor effect partly by restoring the expression levels of miRNAs in blood serum and the hypothalamus, and the gut microbiota composition. Critically, the present *in vitro* study suggests that the combined effects of pachymic acid, saikosaponins a and d, and isoliquiritigenin, which are constituents of YKS, on tumor suppression may explain the promising effect of YKS. This study presents a new approach to evaluate the efficacy of YKS. Further research is

needed to clarify how components of YKS correspond to restoring the levels of miRNAs in blood serum and hypothalamus, and the gut microbiota composition.

## Author contributions

Minoru N designed the research. CH, YH, TK, NK, KA, HM and Michiko N performed experiments. YH, TK, KA, HM and KI analyzed the data. HK, NK, Michiko N and Minoru N supervised the research. CH, MK and Minoru N wrote the paper.

## Compliance with ethical standards

**Conflict of interest**    The authors declare that they have no conflict of interest.

## Reference

1. Sarkar D. K, Murugan S, Zhang C, Boyadjieva N (2012) Regulation of Cancer Progression by β-Endorphin Neuron. Cancer Res 72:836–840 DOI 10.1158/0008-5472.CAN-11-3292

2. Powell N. D, Tarr A. J, Sheridan, J. F (2013) Psychosocial stress and inflammation in cancer. Brain. Behav. Immun 30: 41–47 DIO 10.1016/j.bbi.2012.06.015

3. Cao D. D, Li, L Chan W. Y (2016) MicroRNAs: Key Regulators in the Central Nervous System and Their Implication in Neurological Diseases. Int J Mol Sci 17: 842 DOI 10.3390/ijms17060842

4. Imai S, Saeki M, Yanase M, Horiuchi H, Abe M, Narita M, Kuzumaki N, Suzuki T, Narita M (2011) Change in MicroRNAs Associated with Neuronal Adaptive Responses in the Nucleus Accumbens under Neuropathic Pain. J Neurosci 31: 15294–15299 DOI 10.1523/JNEUROSCI.0921-11.2011

5. Saisu H, Igarashi K, Narita M, Ikegami D, Kuzumaki N, Wajima K, Nakagawa T, Narita M (2015) Neuropathic pain-like stimuli change the expression of ribosomal proteins in the amygdala: genome-wide search for a "pain-associated anxiety-related factor". Jpn J Pharm Palliat Care Sci 8: 47-57

6. Zhang H. F, Xu L. Y, Li E. M (2014) A family of pleiotropically acting microRNAs in cancer progression, miR-200: potential cancer therapeutic targets. Curr Pharm Des 20: 1896–903

7. Narita M, Shimura E, Nagasawa A, Aiuchi T, Suda Y, Hamada Y, Ikegami D, Iwasawa C, Arakawa K, Igarashi K, Kuzumaki N, Yoshioka Y, Ochiya T, Takeshima H, Ushijima T, Narita M (2017) Chronic treatment of non-small-cell lung cancer cells with gefitinib leads to an epigenetic loss of epithelial properties associated with reductions in microRNA-155 and -200c. PLoS One 12: e0172115 DOI 10.1371/journal.pone.0172115

8. Schwabe R. F, Jobin C (2013) The microbiome and cancer. Nat Rev Cancer 13: 800–812 DOI 10.1038/nrc3610

9. Nelson M. H, Diven M. A, Huff L. W, Paulos C. M (2015) Harnessing the Microbiome to Enhance Cancer Immunotherapy. J Immunol Res 368736 DOI 10.1155/2015/368736

10. Zitvogel L, Ayyoub M, Routy B, Kroemer G (2016) Microbiome and Anticancer Immunosurveillance. Cell 165: 276–87 DOI 10.1016/j.cell.2016.03.001

11. Erdman S.E, Poutahidis T (2017) Gut microbiota modulate host immune cells in cancer development and growth. Free Radic Biol Med 105: 28–34 DOI 10.1016/j.freeradbiomed.2016.11.013

12. Ikarashi Y, Mizoguchi K (2016) Neuropharmacological efficacy of the traditional Japanese Kampo medicine yokukansan and its active ingredients. Pharmacol Ther 166: 84–95 DOI 10.1016/j.pharmthera.2016.06.018

13. Yamaguchi T, Tsujimatsu A, Kumamoto H, Izumi T, Ohmura Y, Yoshida T, Yoshioka M (2012) Anxiolytic effects of yokukansan, a traditional Japanese medicine, via serotonin 5-HT1A receptors on anxiety-related behaviors in rats experienced aversive stress. J Ethnopharmacol 143: 533–9 DOI 10.1016/j.jep.2012.07.007

14. Fujiwara H, Han Y, Ebihara K, Awale S, Araki R, Yabe T, Matsumoto K (2017) Daily administration of yokukansan and keishito prevents social isolation-induced behavioral abnormalities and down-regulation of phosphorylation of neuroplasticity-related signaling molecules in mice. BMC Complement Altern Med 17: 195 DOI 10.1186/s12906-017-1710-7

15. Tamano H, Kan F, Oku N, Takeda A (2010) Ameliorative effect of Yokukansan on social isolation-induced aggressive behavior of zinc-deficient young mice. Brain Res Bull 83: 351–5 DOI 10.1016/j.brainresbull.2010.08.013

16. Katahira H, Sunagawa M, Watanabe D, Kanada Y, Katayama A, Yamauchi R, Takashima M, Ishikawa S, Hisamitsu T (2017) Antistress effects of Kampo medicine "Yokukansan" via regulation of orexin secretion. Neuropsychiatr Dis Treat 13: 863–872 DOI 10.2147/NDT.S129418

17. Furuya M, Miyaoka T, Tsumori T, Liaury K, Hashioka S, Wake R, Tsuchie K, Fukushima M, Ezoe S, Horiguchi J (2013) Yokukansan promotes hippocampal neurogenesis associated with the suppression of activated microglia in Gunn rat. J Neuroinflammation 10: 145 DOI 10.1186/1742-2094-10-145

18. Ebisawa S, Andoh T, Shimada Y, Kuraishi Y (2015) Yokukansan Improves Mechanical Allodynia through the Regulation of Interleukin-6 Expression in the Spinal Cord in Mice with Neuropathic Pain. Evid Based Complement Alternat Med 2015 870687 DOI 10.1155/2015/870687

19. Agalioti T, Giannou A.D, Krontira A.C, Kanellakis N.I, Kati D, Vreka M, Pepe M, Spella M, Lilis I, Zazara D.E, Nikolouli E, Spiropoulou N, Papadakis A, Papadia K, Voulgaridis A, Harokopos V, Stamou P, Meiners S, Eickelberg O, Snyder L.A, Antimisiaris S.G, Kardamakis D, Psallidas I, Marazioti A, Stathopoulos G.T. (2017) Mutant KRAS promotes malignant pleural effusion formation. Nat Commun 16:15205 DOI 10.1038/ncomms15205

20. Routy B, Le Chatelier E, Derosa L, Duong C. P. M, Alou M. T, Daillère R, Fluckiger A, Messaoudene M, Rauber C, Roberti M. P, Fidelle M, Flament C, Poirier-Colame V, Opolon P, Klein C, Iribarren K, Mondragon L, Jacquelot L, Qu B, Ferrere G, Clémenson C, Mezquita L, Masip JR, Naltet C, Brosseau S, Kaderbhai C, Richard C, Rizvi H, Rizvi H, Levenez F, Galleron N, Quinquis, B, Pons N, Ryffel B, Minard-Colin V, Gonin P, Soria J.C, Deutsch E, Loriot Y, Ghiringhelli F, Zalcman G,

Goldwasser F, Escudier B, Hellmann M.D, Eggermont A, Raoult D, Albiges L, Kroemer G, Zitvogel L (2017) Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. Science 359: 91-97 DOI 10.1126/science.aan3706

21. Ueki T, Mizoguchi K, Yamaguchi T, Nishi A, Ikarashi Y, Hattori T, Kase Y (2015) Yokukansan Increases 5-HT1A Receptors in the Prefrontal Cortex and Enhances 5-HT1A Receptor Agonist-Induced Behavioral Responses in Socially Isolated Mice. Evid Based Complement Alternat Med 726471 DOI 10.1155/2015/726471

22. Mizoguchi K, and Ikarashi Y (2017) Multiple Psychopharmacological Effects of the Traditional Japanese Kampo Medicine Yokukansan, and the Brain Regions it Affects. Front Pharmacol 8: 149 DOI 10.3389/fphar.2017.00149

23. Nishi A, Yamaguchi T, Sekiguchi K, Imamura S, Tabuchi M, Kanno H, Nakai Y, Hashimoto K, Ikarashi Y, Kase Y (2012) Geissoschizine methyl ether, an alkaloid in Uncaria hook, is a potent serotonin $_1$A receptor agonist and candidate for amelioration of aggressiveness and sociality by yokukansan. Neuroscience 207: 124–36 DOI 10.1016/j.neuroscience.2012.01.037

24. Fiorentino L, Ancoli-Israel S (2007) Sleep dysfunction in patients with cancer. Curr Treat Options Neurol 9: 337–46

25. Vétizou M, Pitt J. M, Daillère R, Lepage P, Waldschmitt N, Flament C, Rusakiewicz S, Routy B, Roberti M. P, Duong C. P, Poirier-Colame V, Roux A, Becharef S, Formenti S, Golden E, Cording S, Eberl G, Schlitzer A, Ginhoux F, Mani S, Yamazaki T, Jacquelot N, Enot D. P, Bérard M, Nigou J, Opolon P, Eggermont A, Woerther P. L, Chachaty E, Chaput N, Robert C, Mateus C, Kroemer G, Raoult D, Boneca I. G, Carbonnel F, Chamaillard M. Zitvogel L (2015) Anticancer

immunotherapy by CTLA-4 blockade relies on the gut microbiota. Science 350: 1079–84 DOI 10.1126/science.aad1329

26. <u>Wu M, Wu Y, Deng B, Li J, Cao H, Qu Y, Qian X, Zhong G (2016) Isoliquiritigenin decreases the incidence of colitis-associated colorectal cancer by modulating the intestinal microbiota. Oncotarget 7: 85318-85331 DOI 10.18632/oncotarget.13347</u>

27. Ciardiello F, Tortora G (2003) Epidermal growth factor receptor (EGFR) as a target in cancer therapy: understanding the role of receptor expression and other molecular determinants that could influence the response to anti-EGFR drugs. Eur J Cancer 39: 1348–54

28. Forcella M, Oldani M, Epistolio S, Freguia S, Monti E, Fusi P, Frattini M (2017) Non-small cell lung cancer (NSCLC), EGFR downstream pathway activation and TKI targeted therapies sensitivity: Effect of the plasma membrane-associated NEU3. PLoS One 12: e0187289  DOI 10.1371/journal.pone.0187289

29. Morodomi Y, Okamoto T, Maehara Y (2017) Reply to "EGFR Mutation in Patients with Lung Adenosquamous Cell Carcinoma". Ann Surg Oncol 24(3): 676 DOI 10.1245/s10434-017-6181-z

30. Bethune G, Bethune D, Ridgway N, Xu Z (2010) Epidermal growth factor receptor (EGFR) in lung cancer: an overview and update. J Thorac Dis 2: 48–51

31. Pancewicz-Wojtkiewicz J (2016) Epidermal growth factor receptor and notch signaling in non-small-cell lung cancer. Cancer Med 5: 3572-3578 DOI 10.1002/cam4.944

32. Zhang W, Liu H. T (2002) MAPK signal pathways in the regulation of cell proliferation in mammalian cells. Cell Res 12: 9–18

33. Brantley E. C, Benveniste E. N (2008) Signal Transducer and Activator of Transcription-3: A Molecular Hub for Signaling Pathways in Gliomas. Mol Cancer Res 6: 675–684 DOI 10.1158/1541-7786.MCR-07-2180

34. Fung T. C, Olson C. A, Hsiao E. Y (2017) Interactions between the microbiota, immune and nervous systems in health and disease. Nat Neurosci 20: 145–155. DOI 10.1038/nn.4476

35. Meister B, Herzer S, Silahtaroglu A (2013) MicroRNAs in the Hypothalamus. Neuroendocrinology 98: 243–253 DOI 10.1159/000355619

36. Geaghan M, Cairns M. J (2015) MicroRNA and Posttranscriptional Dysregulation in Psychiatry. Biol. Psychiatry 78: 231–9 DOI 10.1016/j.biopsych.2014.12.009

37. Jin J, Cheng Y, Zhang Y, Wood W, Peng Q, Hutchison E, Mattson M. P, Becker K.G, Duan W (2012) Interrogation of brain miRNA and mRNA expression profiles reveals a molecular regulatory network that is perturbed by mutant huntingtin. J Neurochem 123: 477–90 DOI 10.1111/j.1471-4159.2012.07925.x

38. Wang Y, Wang Y, Yang G. Y (2013) MicroRNAs in Cerebral Ischemia. Stroke Res Treat 2013: 276540 DOI 10.1155/2013/276540

39. Zhu J, Chen Z, Tian J, Meng Z, Ju M, Wu G, Tian Z (2017) miR-34b attenuates trauma-induced anxiety-like behavior by targeting CRHR1. Int J Mol Med 40: 90–100 DOI 10.3892/ijmm.2017.2981

40. Li C, Liu Y, Liu D, Jiang H, Pan F (2016) Dynamic Alterations of miR-34c Expression in the Hypothalamus of Male Rats after Early Adolescent Traumatic Stress. Neural Plast 2016: 5249893 DOI 10.1155/2016/5249893

41. Jadhav S. P, Kamath S. P, Choolani M, Lu J, Dheen S. T (2014) microRNA-200b modulates microglia-mediated neuroinflammation via the cJun/MAPK pathway. J Neurochem 130: 388–401   DOI 10.1111/jnc.12731

42. Beclin C, Follert P, Stappers E, Barral S, Nathalie C, de Chevigny A, Magnone V, Lebrigand K, Bissels U, Huylebroeck D, Bosio A, Barbry P, Seuntjens E, Cremer H (2016) miR-200 family controls late steps of postnatal forebrain neurogenesis via Zeb2 inhibition. Sci Rep 6: 35729 DOI 10.1038/srep35729

43. Gapter L, Wang Z, Glinski J, NG K. Y (2005) Induction of apoptosis in prostate cancer cells by pachymic acid from Poria cocos. Biochem Biophys Res Commun 332(4): 1153-1161

44. Ling H, Zhang Y, Ng K. Y, Chew E.H (2011) Pachymic acid impairs breast cancer cell invasion by suppressing nuclear factor-kappaB-dependent matrix metalloproteinase-9 expression. Breast Cancer Res Treat 126(3): 609-620 DOI 10.1007/s10549-010-0929-5

45. Chen S. Swanson K, Eliaz I, McClintick J. N, Sandusky G. E, Sliva D (2015) Pachymic acid inhibits growth and induces apoptosis of pancreatic cancer in vitro and in vivo by targeting ER stress. PLoS One 10(4): e0122270 DOI 10.1371/journal.pone.0122270.

46. Ma J, Liu J, Lu C, Cai D (2015) Pachymic acid induces apoptosis via activating ROS-dependent JNK and ER stress pathways in lung cancer cells. Cancer Cell Int 15: 78 DOI 10.1186/s12935-015-0230-0

47. Lu, C, Ma J, Cai D (2017) Pachymic acid inhibits the tumorigenicity of gastric cancer cells by the mitochondrial pathway. Anticancer Drugs 28(2): 170-179 DOI 10.1097/CAD.0000000000000449.

48. Kim B. M, S. H. Hong (2011) Sequential caspase-2 and caspase-8 activation is essential for saikosaponin a-induced apoptosis of human colon carcinoma cell lines. Apoptosis 16(2): 184-197 DOI 10.1007/s10495-010-0557-x

49. Chen J. C, Chang N. W, Chung J. G, Chen K. C (2003) Saikosaponin-A induces apoptotic mechanism in human breast MDA-MB-231 and MCF-7 cancer cells. Am J Chin Med 31(3): 363-377

50. Hsu Y. L, Kuo P. L, Chiang L. C, Lin C. C (2004) Involvement of p53, nuclear factor kappaB and Fas/Fas ligand in induction of apoptosis and cell cycle arrest by saikosaponin d in human hepatoma cell lines. Cancer Lett 213(2): 213-221

51. Hsu Y. L, Kuo P. L, Lin C. C (2004). The proliferative inhibition and apoptotic mechanism of Saikosaponin D in human non-small cell lung cancer A549 cells. Life Sci 75(10): 1231-1242

52. Liu, R. Y, J. P. Li (2014) Saikosaponin-d inhibits proliferation of human undifferentiated thyroid carcinoma cells through induction of apoptosis and cell cycle arrest. Eur Rev Med Pharmacol Sci 18(17): 2435-2443

53. Li Y, Cai T, Zhang W, Zhu W, Lv S (2017) Effects of Saikosaponin D on apoptosis in human U87 glioblastoma cells. Mol Med Rep 16(2): 1459-1464 DOI 10.3892/mmr.2017.6765

54. Hsu Y. L, Kuo P. L, Chiang L. C, Lin C. C (2004) Isoliquiritigenin inhibits the proliferation and induces the apoptosis of human non-small cell lung cancer a549 cells. Clin Exp Pharmacol Physiol 31(7): 414-418

55. Ii T, Satomi Y, Katoh D, Shimada J, Baba M, Okuym Y, Nishino H, Kitamura N (2004) Induction of cell cycle arrest and p21(CIP1/WAF1) expression in human lung cancer cells by isoliquiritigenin. Cancer Lett 207(1): 27-35

56. Jung S. K, Lee M. H, Lim D. Y, Kim J. E, Singh P, Lee S. Y, Jeong C. H, Lim T. G, Chen H, Chi Y. I, Kundu J. K, Lee N. H, Lee C. C, Cho Y. Y, Bode A. M, Lee K. W, Dong Z (2014). Isoliquiritigenin induces apoptosis and inhibits xenograft tumor growth of human lung cancer cells by targeting both wild type and L858R/T790M mutant EGFR. J Biol Chem 289(52): 35839-35848 DOI 10.1074/jbc.M114.585513

**Figure Legends**

**Fig. 1** Time-course of tumor volume, food intake and body weight of mice fed pellets containing YKS. (A) Experimental schedule. (B) Quantitative analysis of tumor size in tumor-bearing mice fed control food (CF-LLC) (n=12) and control food containing 0.6% YKS-LLC (YKSF-LLC) (n=14) at 4, 7, 11, 14, 18, and 21 days after tumor implantation (***$p<0.001$ vs. CF-LLC). (C, D) Time-course of changes in food intake (C) and body weight (D) of LLC-bearing mice. All data are presented as means ± SEM.

**Fig. 2** Effect of YKSF on the gut microbiota composition. (A) Unsupervised hierarchical clustering of normalized read number of each microbiota species rRNA DNA amplicons in each mouse. Values at branches are approximately unbiased (AU) *P* values (%) computed by bootstrap resampling. Red and blue colors presented in the heatmap indicate values above and below the mean value of each microbiota species in each mouse, respectively. Yellow indicates values close to the mean. Grey indicates values between minimum (blue) and medium (yellow). (B) Changes in the fecal microbiota composition of non-tumor (NT) and LLC-bearing (LLC) mice at 22 days of feeding with CF or YKSF. Columns show means ± SEM. *$p < 0.05$, **$p < 0.01$ vs. NT-CF group, #$p < 0.05$ vs. LLC-CF group.

**Fig. 3** Effect of YKSF on the expression of miRNAs in blood serum. (A, B) Pearson's correlation scatterplot of miRNA levels in control food-LLC bearing mice (CF-LLC) vs. control food-non-tumor mice (CF-NT) (A), and control food containing 0.6%YKS-LLC bearing mice (YKSF-LLC) vs. CF-NT mice (B) at 22 days. The blue points represent miRNAs for which expression in the CF-LLC group was two-fold more or less than that in the CF-NT group, and for which expression in the YKSF-LLC group was within two-fold of that in the CF-NT group. (C) Relative expression of miR-133a-3p, miR-133b-3p, miR-1a-3p and miR-342-3p in blood serum of NT and LLC mice at 22 days after YKS treatment by miRNA array. (D) Venn diagram of the results of a search for target mRNA candidates for miR-133a-3p/133b-3p, miR-1a-3p and miR-342-3p by a TargetScan analysis. The total number of target mRNA candidates is 1475. (E) Top 10 pathways extracted from a Wikipathway analysis. All of the target mRNA candidates as input are shown. The yellow pathway is the EGFR1 Signaling Pathway. (F) Diagram of the EGFR1 signaling pathway (WP572_71756). mRNAs in red boxes are matched mRNAs.

**Fig. 4** Effect of YKSF on the expression of miRNAs in the hypothalamus. Quantitative analysis of the expression of miR-200b-3p (A), miR-200c-3p (B), miR-429-3p (C), miR-34c-3p (D), and miR-34b-5p (E) in the hypothalamus of NT and LLC mice at 22 days after YKS treatment. Columns represent means ± SEM. *$p < 0.05$, **$p<0.01$, ***$p < 0.001$ vs. NT-CF group, #$p < 0.05$ vs. LLC-CF group.

**Fig. 5** Compounds in YKS that inhibit LLC cell proliferation. (A) LLC cells were incubated with each of the seven compounds (10 µM) contained in YKS, i.e., pachymic acid, palmitic acid, saikosaponins a, c and d, β-eudesmol, and isoliquiritigenin, for 48 hr, and cell viability was determined by the MTT assay. (B) LLC cells were incubated with pachymic acid, saikosaponins a and d, and isoliquiritigenin (1 and 3 µM), and a mixture of these 4 compounds, for 48 hr, and cell viability was determined by the MTT assay. Columns represent means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. control.

**Table 1.** Quantitative validation of the expression changes in circulating miRNAs in the serum in Figure 3C.

Fig. 1
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**A**

YKSF-LLC No.1
YKSF-LLC No.2
CF-LLC No.1
CF-LLC No.2
YKSF-NT No.1
YKSF-NT No.3
YKSF-LLC No.3
CF-NT No.1
CF-LLC No.3
YKSF-NT No.2
CF-NT No.2
CF-NT No.3

Relative abundance

High          Low

**B**

*Bacteroidales*

** #

Relative expression

CF   YKSF   CF   YKSF
NT          LLC

*Desulfovibrionaceae*

** #

Relative expression

CF   YKSF   CF   YKSF
NT          LLC

*Veillonellaceae*

*

Relative expression

CF   YKSF   CF   YKSF
NT          LLC

Fig. 2
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**A**

CF-LLC

CF-NT

[Cont, Cont]

**B**

YKSF-LLC

CF-NT

[Cont, Cont]

Color by [Cont, Cont]

0    9.2

**Total: 1475**

**C**

miR-133a-3p

Relative expression

| CF | YKSF | CF | YKSF |
| NT | | LLC | |

miR-133b-3p

Relative expression

| CF | YKSF | CF | YKSF |
| NT | | LLC | |

miR-1a-3p

Relative expression

| CF | YKSF | CF | YKSF |
| NT | | LLC | |

miR-342-3p

Relative expression

| CF | YKSF | CF | YKSF |
| NT | | LLC | |

**D**

miR-133a-3p/133b-3p
Targets: 616

miR-1a-3p
Targets: 729

521   69   624

18   8   28

207

miR-342-3p
Targets: 261

**E**

| Pathway | p-value | Matched Entities | Pathway Entities |
|---|---|---|---|
| Mm_XPodNet_–_protein– protein_interactions_in_the_podocyte_ expanded_by_STRING_WP2309_72004 | 0 | 133 | 836 |
| Mm_PodNet_–_protein– protein_interactions_in_the_podocyte_ WP2310_72005 | 5.52E–11 | 62 | 315 |
| Mm_PluriNetWork_WP1763_72003 | 1.06E–05 | 42 | 291 |
| Mm_TGF– beta_Receptor_Signaling_Pathway_WP 258_73847 | 2.48E–05 | 26 | 150 |
| Mm_Wnt_Signaling_Pathway_NetPath_ WP539_71716 | 2.81E–05 | 21 | 109 |
| Mm_Regulation_of_Actin_Cytoskeleton _WP523_71326 | 1.83E–04 | 24 | 151 |
| Mm_EGFR1_Signaling_Pathway_WP57 2_71756 | 3.77E–04 | 26 | 176 |
| Mm_Wnt_Signaling_Pathway_and_Pluri potency_WP723_69165 | 3.90E–04 | 17 | 97 |
| Mm_Endochondral_Ossification_WP12 70_72216 | 4.26E–04 | 13 | 62 |
| Mm_mRNA_processing_WP310_71745 | 5.29E–04 | 51 | 549 |

**F**

Fig. 3
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Fig. 4
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**(A)**

MTT assay



**(B)**

MTT assay



Fig. 5

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Table 1. Quantitative validation of the expression changes in circulating miRNAs in the serum in Figure 3C

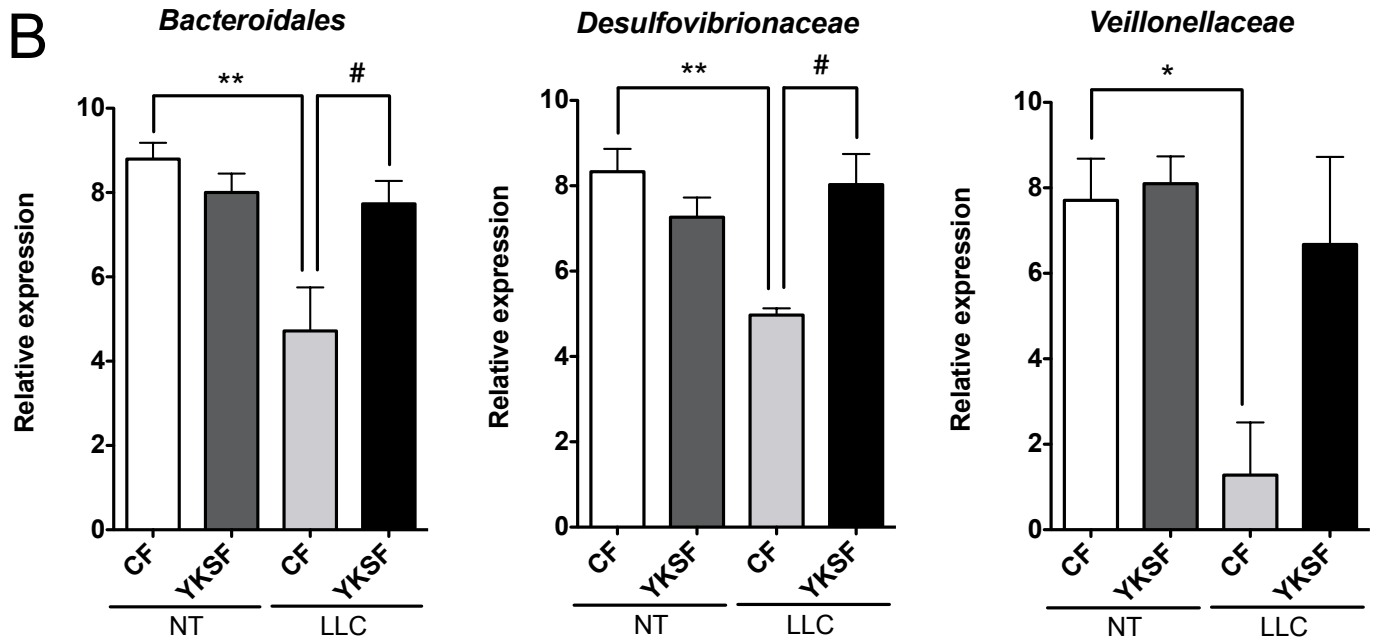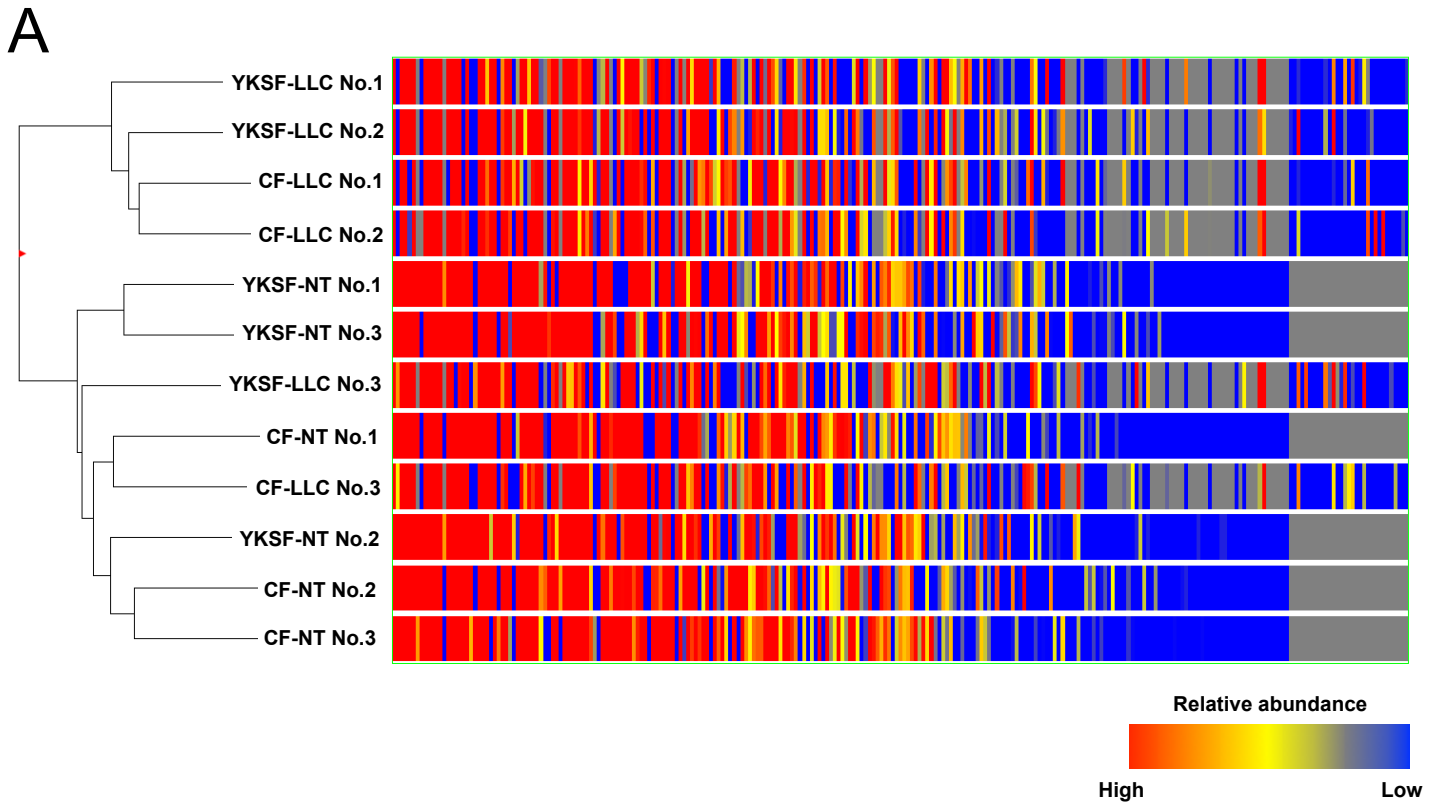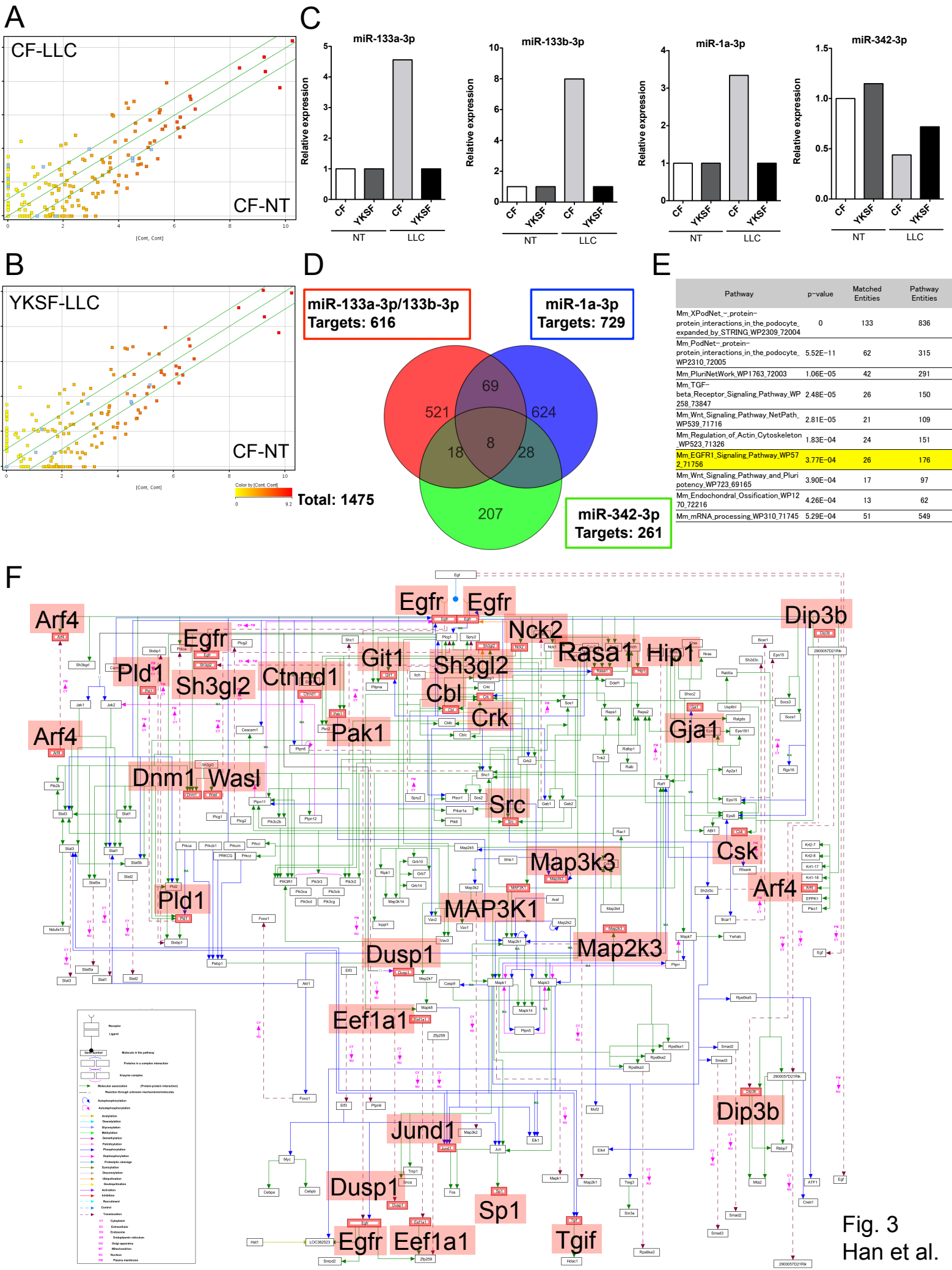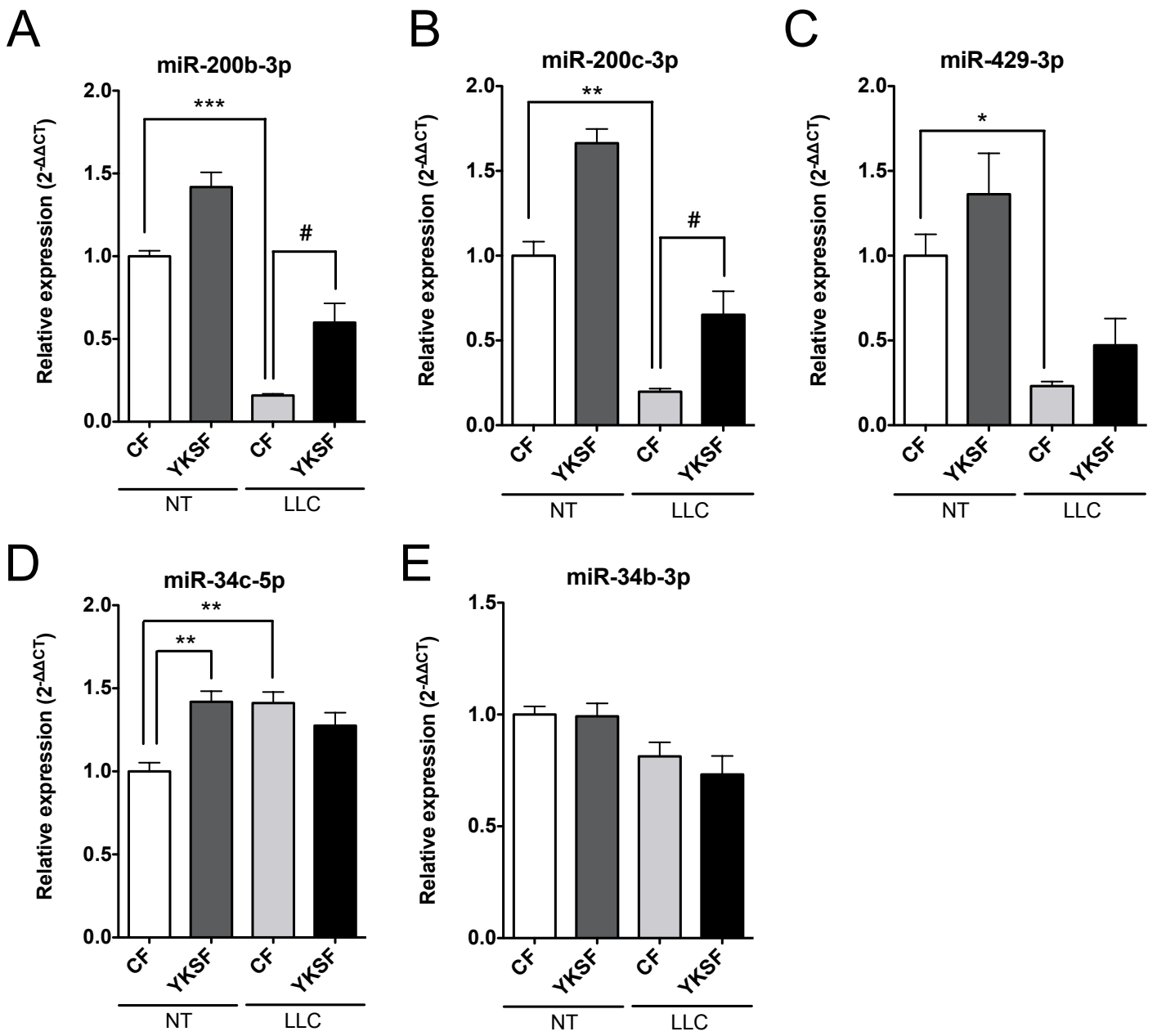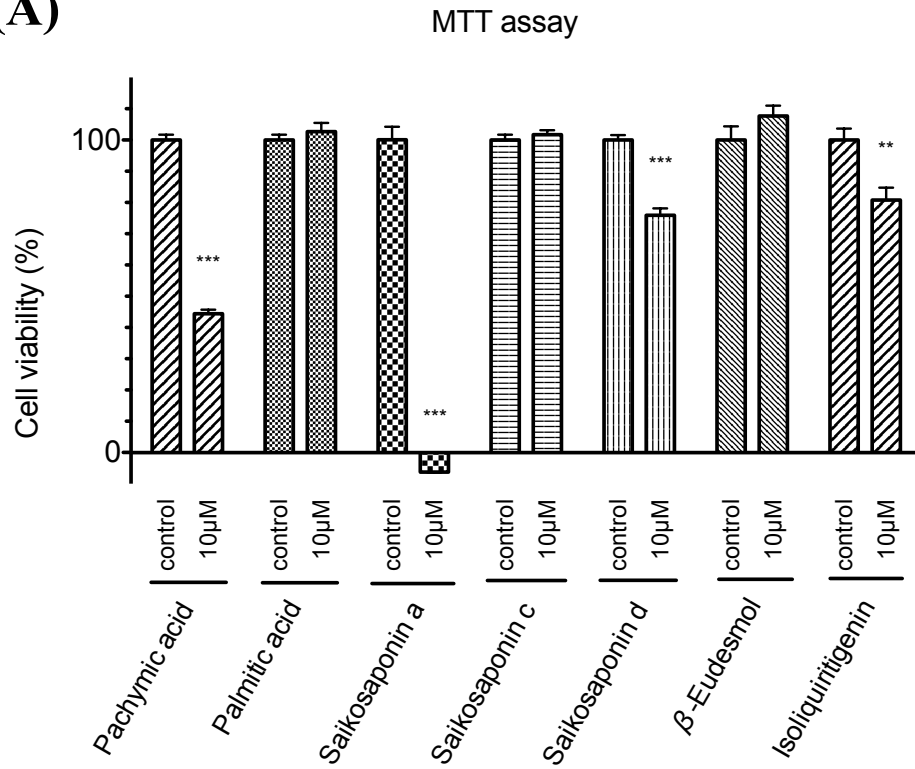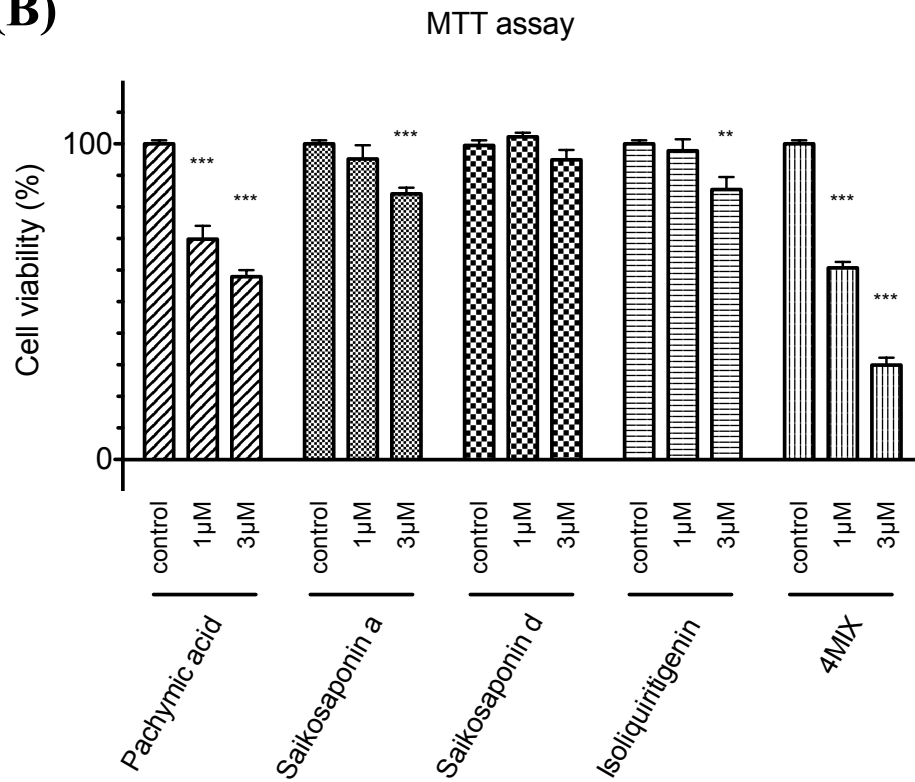| Target miRNA | Sample | Av. ΔCT | Fold change (vs. CF-NT) | S.E.M. | p-value |
|---|---|---|---|---|---|
| miR-133a-3p | CF-NT | N.D. | - | - | - |
| | YKSF-NT | N.D. | - | - | - |
| | CF-LLC | 5.75 | - | 0.297 | - |
| | YKSF-LLC | N.D. | - | - | - |
| miR-133b-3p | CF-NT | N.D. | - | - | - |
| | YKSF-NT | N.D. | - | - | - |
| | CF-LLC | 5.55 | - | 0.109 | - |
| | YKSF-LLC | N.D. | - | - | - |
| miR-1a-3p | CF-NT | N.D. | - | - | - |
| | YKSF-NT | N.D. | - | - | - |
| | CF-LLC | 5.99 | - | 0.064 | - |
| | YKSF-LLC | N.D. | - | - | - |
| miR-342-3p | CF-NT | -4.90 | 1.00 | 0.076 | - |
| | YKSF-NT | -5.11 | 1.16 | 0.104 | P=0.2560 (vs. CF-NT) |
| | CF-LLC | -3.73 | 0.448 | 0.043 | P=0.0007*** (vs. CF-NT) |
| | YKSF-LLC | -4.37 | 0.702 | 0.075 | P=0.0260* (vs. CF-LLC) |

**Abbreviations:** N.D., not detected.                                                    n=3-4.

Table 1

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