Note

Anti-tumor effect of *Inonotus obliquus* in xenograft animals with EBV+human gastric carcinoma

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**ABSTRACT:** *Inonotus obliquus* is a medicinal mushroom with a variety of biological activities. It has reported to have strong anti-cancer, antioxidant and anti-inflammatory properties. EBV+ gastric carcinoma is one of the most common EBV-associated cancers that were caused by latent EBV infection. In this study, we investigated the anti-cancer effects of ethanol extract of *I. obliquus* using *in vivo* xenograft animal models implanted with EBV+ human gastric carcinoma [SNU719]. We also explored the molecular mechanisms responsible for its anti-cancer activity. The result indicated that the extract of *I. obliquus* had an anti-cancer effect in *in vivo* xenograft mice with EBV+ gastric carcinoma [SNU719]. Extract of *I. obliquus* also showed a great effect on inducing the expression of p53, p21 and Bax in tumor tissue derived from EBV+ human gastric carcinoma, and these were correlated with increased expressions of the cleaved forms of caspase-9 and Parp. Also, *I. obliquus* attenuated the expression of viral proteins, BZLF-1 and LMP-2 in tumor tissue from EBV+ human gastric carcinoma.

**Key words:** *Inonotus obliquus*, EBV+human gastric carcinoma, caspase-9, p53, PARP
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10% heat-inactivated fetal bovine serum (Hyclone), 100 U/ml penicillin/streptomycin (Gibco) at 37°C in a humidified 5% CO2 air atmosphere. Animal experiment was conducted in accordance with the National Research Council’s Guide (IACUC, Republic of Korea) for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Animal Experiments Committee of Duksung Women’s University. NOD/SCID mice (female, 5 weeks old; Raonbio Co. Ltd.) were used as xenograft animal models. Mice were individually accommodated in a pathogen free controlled environment (23–27°C under a 12 h day/12 h night cycle) and provided food and water ad lib.

To investigate the anti-tumor effect of ethanol extract of I. obliquus (IO) in vivo, xenograft mice were randomly divided into two groups and subcutaneously injected with EBV+ gastric carcinoma cells (5 × 10^6 cells/mouse), SNU719, into the dorsum next to the right hind leg. After 2 weeks, each group was orally administered drinking water or ethanol extract of I. obliquus (IO) (30 mg/kg) for 2 weeks. Tumors were identified and measured every other day using a standard caliper; tumor size was calculated using [tumor length (mm) × tumor width (mm)]^2/2 as previously described (Lee et al., 2015a, 2015b). After tumor size had reached 2,000 mm^3, animals were euthanized and tumors were harvested.

Figure 1 shows that IO extract inhibited the growth of EBV+ human gastric carcinoma (SNU719) on day 15 (DW; 467.1 mm^3, IO extract; 328.8 mm^3) through day 17 (DW; 469.6 mm^3, IO extract; 332.1 mm^3), and the growth inhibition was significant compared to drinking water group.

Nakata group informed that inotodiol from I. obliquus mediates anti-tumor promoting activity in vivo carcinogenesis test (Nakata et al., 2007). Therefore, we speculated the anti-tumor effect of I. obliquus in EBV+ human gastric carcinoma.

Next, the molecular mechanisms for anti-cancer activity of I. obliquus (IO) have been examined. p53 and Bax play critical roles in cell apoptosis and p21 is a key factor in cell cycle regulation (Li et al., 2012). Therefore, tumor tissues were harvested from three groups of animals bearing EBV+ human gastric carcinoma and then lysed using buffer solution. The expression levels of p53, p21 and Bax were then assessed in lysate proteins from each group. We found increased expressions of p53, p21 and Bax in EBV+ human gastric carcinoma bearing animals fed with IO extract (Fig. 2A and B). In fact, Inotodiol, one of the major compounds from I. obliquus was shown to induce the apoptosis of A549 cell lines through the up-regulation of p53 and Bax expression (Zhong et al., 2011). The induction of cell apoptosis includes not only p53-related signaling but also the activations of caspases and poly ADP-ribose polymerase (Parp) (Koh et al., 2005). Caspases start with two different apoptotic pathways, the extrinsic and intrinsic pathways. The intrinsic pathway is triggered by endogenous stimuli, such as DNA damage and oxidative stress and mainly signals through activated form of cleaved caspase-9 (Matt and Hofmann, 2016). In Fig. 3, there is a dramatic increase of caspase-9 expression in EBV+ human gastric carcinoma from animals fed with IO extract compared to animals fed with drinking water. Inotodiol-rich extract was reported to inhibit cell proliferation through apoptosis induction by activating caspase-3 (Nomura et al., 2008). Therefore, we suggest that I. obliquus induces the intrinsic pathway of apoptosis through the activation of caspase-9 and -3. Of note, IO extract significantly amplified the expressions of the cleaved forms of caspase-9 (Fig. 3A), which reflects our in vivo results in Fig. 1. Poly ADP-ribose polymerase (Parp) is a family of proteins involved in DNA repair and programmed cell death. Cleavage of Parp by caspases is typically known to inactivate Parp activity (Koh et al., 2005). In Fig. 3B, we found that the expressions of cleaved Parp were clearly upregulated in EBV+ human gastric carcinoma bearing animals fed with IO extract. Importantly, IO extract...
**Fig. 2.** Expression of p53, p21 and Bax in tumor tissues from mice implanted with EBV+ human gastric carcinoma (SNU719). EBV+ human gastric carcinoma tumor tissue was excised from each animal fed *Inonotus obliquus* extract (IO) or drinking water (Non.) and prepared for western blot analysis. The protein expressions of (A) p53, p21 and (B) Bax were identified and the relative intensities were measured. β-Actin was used as the loading control.

**Fig. 3.** Expressions of (cleaved) caspase-9 and (cleaved) Parp proteins in tumor tissues from mice implanted with EBV+ human gastric carcinoma (SNU719). EBV+ human gastric carcinoma tumor tissue was excised from each animal fed *Inonotus obliquus* extract (IO) or drinking water (Non.) and prepared for western blot analysis. The expressions of (A) (cleaved) caspase-9 and (B) (cleaved) parp were identified and relative intensities were measured. β-Actin was used as the loading control.
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Fig. 4. Expressions of BZLF-1 and LMP-2 proteins in tumor tissues from mice implanted with EBV+ human gastric carcinoma (SNU719). EBV+ human gastric carcinoma tumor tissue was excised from each animal fed I. obliquus extract (IO) or drinking water (Non.) and prepared for western blot analysis. The protein expressions of BZLF-1 and LMP-2 were identified and relative intensities were measured. β-Actin was used as the loading control.

extract intensely increased the expression of cleaved Parp (Fig. 3B).

In addition, we analyzed the expression of EBV proteins (BZLF-1 and LMP-2) in EBV+ tumor tissues derived from IO extract and drinking water fed group by performing Western blot assays using anti-BZLF-1 and LMP-2 antibodies (Fig. 4). EBV BZLF-1 is a key factor for EBV lytic reactivation and LMP-2 protein is known to be essential for EBV latency (Lee et al., 2015c). As shown in Fig. 4, the expressions of BZLF-1 and LMP-2 were moderately repressed by IO extract, suggesting IO extract has a potential for anti-EBV effect. In fact, Taji group reported that triterpenoids from I. obliquus had inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) (Nakata et al., 2007; Taji et al., 2008). In summary, our study is the first report to show the anti-tumor effect of I. obliquus using in vivo xenograft animals bearing human gastric carcinoma, especially in presence of EBV.

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References


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