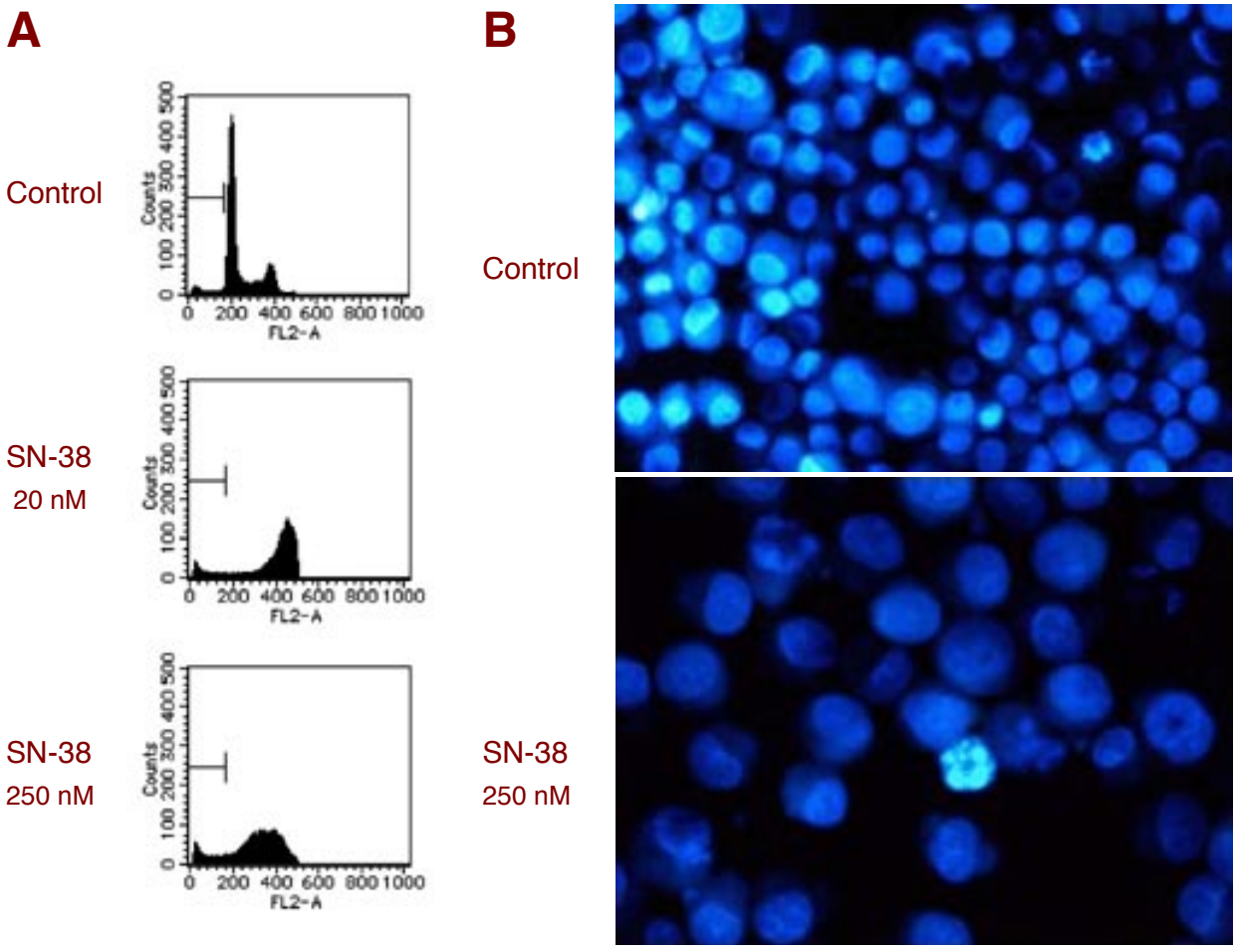


Fig. 3. Influence of SN-38 to MKN1 cells.
A: Effect of 20 nM or 250 nM SN-38 on the cell cycle progression of MKN1 cells after 48 h incubation determined by flow cytometry.
B: Fluorescence microscopy dyeing, Hoechst 33342 after 250 nM SN-38 treatment for 48 h.

PI3K inhibitor reduced the expression level of pAkt and induced apoptosis in MKN1 cells

Through SN-38 treatment, pAkt expression in MKN45 cells was reduced but not in MKN1 cells while MKN45 cells have a higher sensitivity to SN-38 than MKN1 cells. To elucidate that the PI3K-Akt pathway is involved in SN-38-induced apoptosis, we examined whether LY294002 could affect SN-38-induced apoptosis in MKN1 cells. LY294002 is a specific PI3K inhibitor that has been widely used to study the role of PI3K in various biological responses.

The expression level of pAkt in MKN1 cells reduced in an SN-38 dose-dependent manner after

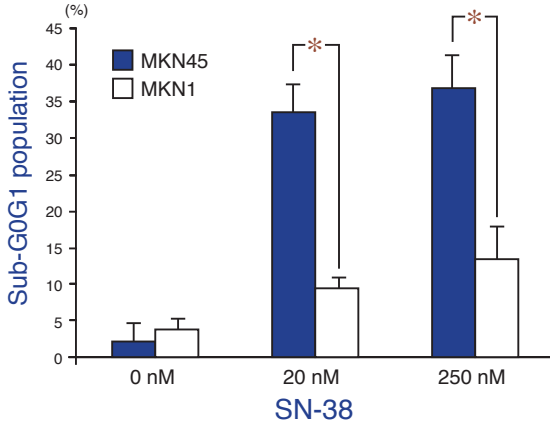


Fig. 4. Sub-G0G1 population in MKN45 and MKN1 cells treated with 0, 20 or 250 nM SN-38 for 48 h. *$P < 0.05$ using Student's *t*-test.

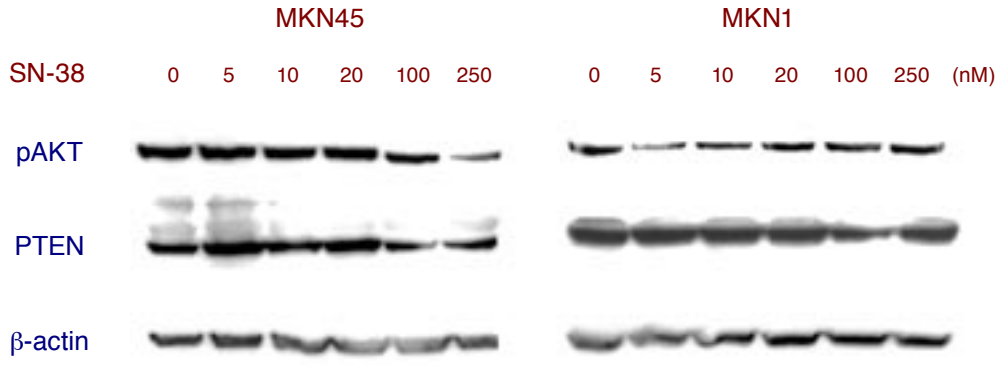


Fig. 5. Western blotting of pAkt and PTEN expression after 0, 5, 10, 20, 100 or 250 nM SN-38 treatment for 48 h.

pretreatment with 50 μM LY294002 (Fig. 6). We also examined PTEN expression using Western blotting, but LY294002 had no influence on PTEN expression (Fig. 6).

Moreover, by cell cycle analysis, the apoptotic sub-G1 fraction appeared by flow cytometry (Fig. 7A). By morphological examination, apoptotic cells were increased by treatment with SN-38 and LY294002 (Fig. 7B). The apoptotic sub-G1 fraction of the population of MKN1 cells was 35.9% 48 h after treatment with 250nM SN-38 and 50 μM LY294002 (Fig. 8). Exposure to 50 μM LY294002 increased SN-38-induced apoptosis in MKN1 cells to as much as that in MKN45 cells after treatment with only SN-38 (Fig. 8). These results indicated that the PI3K-Akt pathway plays a significant role in SN-38-induced apoptosis in human gastric cancer cells.

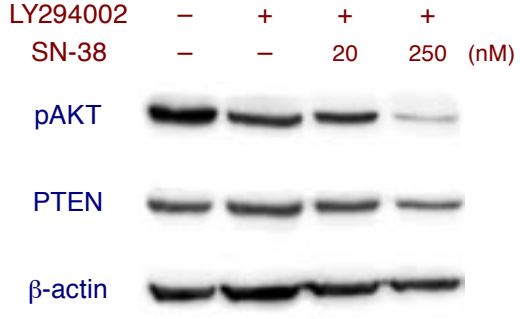
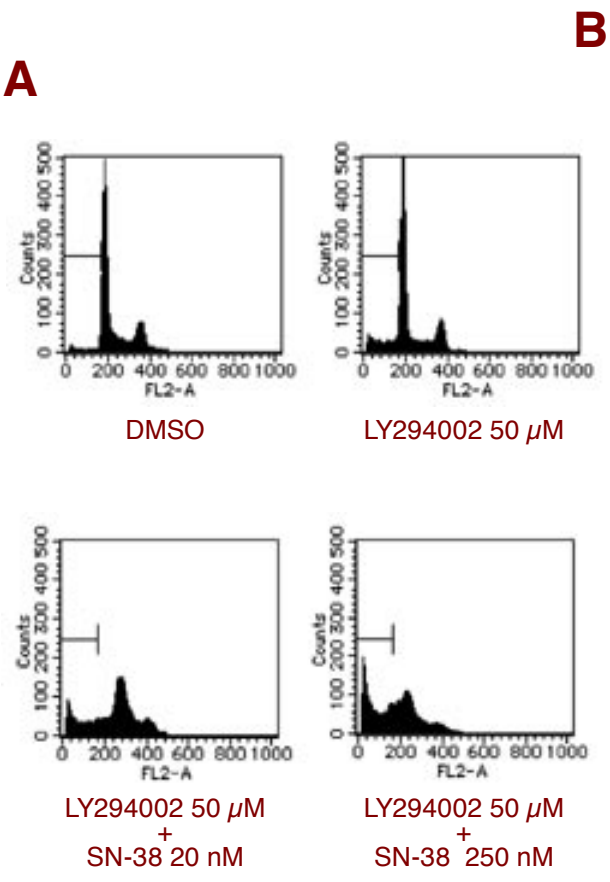


Fig. 6. Western blot of pAkt and PTEN expression. MKN1 cells were treated with 0, 20 or 250 nM SN-38 for 48 h in the presence of 50 μM LY294002.

Discussion

The MKN45 cells were more sensitive to SN-38 than the MKN1 cells. The difference of sensitivity mainly comes from the percentage of apoptotic cells. The pattern of tumor response after in vivo topotecan treatment was reported to correlate with the ability of the drug to induce apoptosis but not with its in vitro antiproliferative activity (Caserini et al., 1997). Apoptosis is a major mode of cell death in response to chemotherapeutic drug treatment (Hickman, 1992; Kerr et al., 1994), and resistance to apoptosis induction has been proposed as a critical mechanism in drug resistance (Kataoka et al., 1994; Chen et al., 1996; Seimiya et al., 1997). However, the mechanisms by which chemotherapeutic agents induce apoptosis are not fully understood. The sensitivity of cells to chemotherapeutic drug-induced apoptosis appears to depend on the balance between proapoptotic and antiapoptotic signals. Therefore, it is possible that a chemotherapeutic drug may induce apoptosis not only by increasing the proapoptotic signals but also by decreasing the antiapoptotic ones.

The PI3K-Akt signaling pathway is known to transmit survival signals and to protect a variety of cells from apoptosis. Akt plays a critical role in controlling the balance between cell survival and apoptosis (Balendran et al., 1999). Phosphorylation of Akt is promoted by phosphatidylinositides converted by PI3K products. Previous reports have



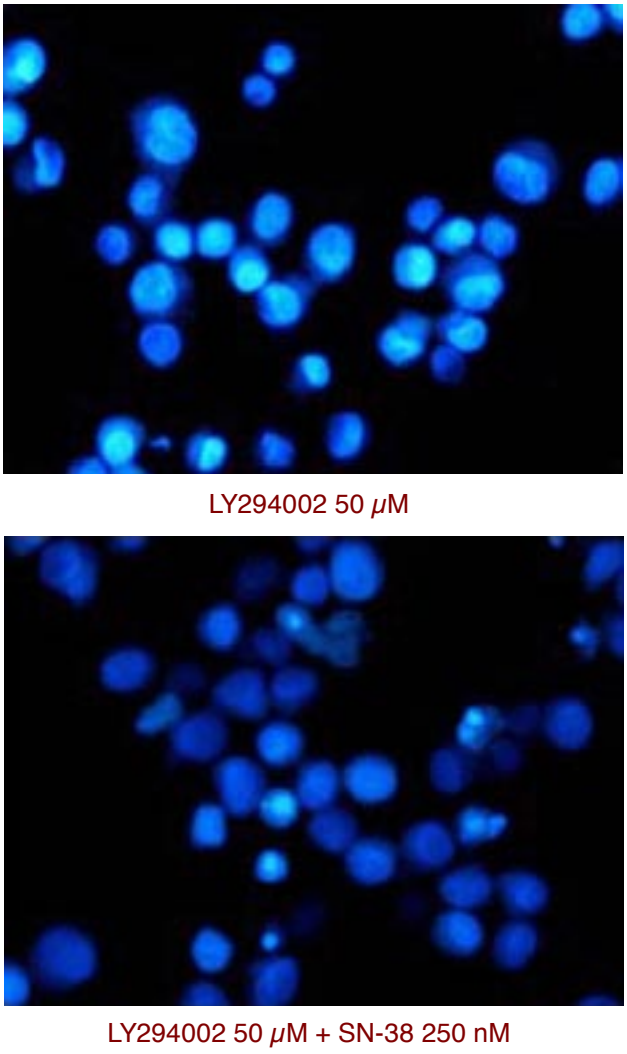


Fig. 7. Influence of LY294002 to MKN1cells.
A: Cell cycle analysis by flow cytometry.
B: Morphological detection of apoptosis by Hoechst 33342 staining.

shown that Akt delivers antiapoptotic survival signals by phosphorylating Bad and activating caspase-9 (Cardone et al., 1998; Brunet et al., 1999). In addition, inhibition of Akt signaling can induce apoptosis in some human cancer cell lines (Page et al., 2000; Yuan et al., 2000). It has also been reported that Akt plays an important role in cell survival when cells are exposed to different apoptotic stimuli, such as growth factor withdrawal, ultraviolet radiation, matrix detachment, cell cycle discordance and DNA damage (Hemmings, 1997; Kulik et al., 1997; Kulik and Weber, 1998; Ng et al., 2000; Brognard et al., 2001). Moreover, inactivation of PI3K using a specific inhibitor has led to dephosphorylation of Akt at Ser473, consequently causing translocation of Akt to nuclei,

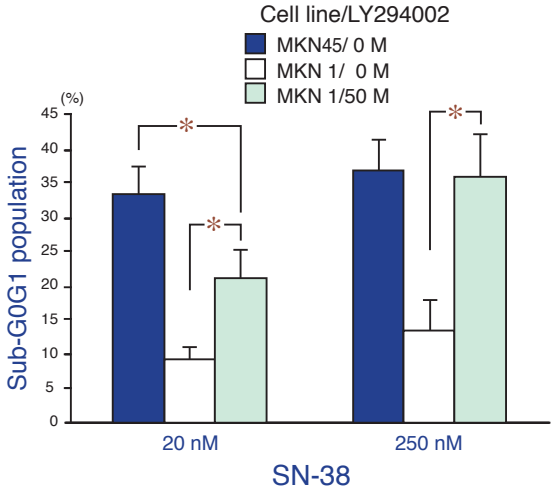


Fig. 8. Sub-G0G1 population in MKN45 and MKN1 cells treated with 0, 20 or 250 nM SN-38 in the presence or absence of LY294002 for 48 h. *$P < 0.05$ using Student's *t*-test.

where it is believed to regulate the transcription of genes mediating cell survival or apoptosis (Hemmings, 1997). In a previous study, TRAIL and Fas induced apoptosis by inhibition of the PI3K-Akt pathway in human gastric cancer cells (Nam et al., 2003; Osaki et al., 2004). Our study showed that apoptosis was induced in cells where pAkt expression was reduced (MKN45), but not in cells where pAkt expression showed no change (MKN1) by SN-38. It has been reported that exposure to camptothecin induced a dose-dependent trapping in the G2/M phase affects and dose-dependent delay in the S phase. Treatment of MKN1 cells with 48 h 20 nM SN-38 induced G2/M phase arrest, and 48 h 250 nM SN-38 treatment induced S phase delay. Meanwhile in MKN45 cells, 48 h 20 nM SN-38 treatment induced G2/M phase arrest tendency and 48 h 250 nM SN-38 treatment induced sub-G1 peak. We speculate that low dose SN-38 treatment below 20 nM induces the G2/M arrest in MKN45 cells and 20 nM SN-38 treatment begins to induce S phase cells. Moreover, in MKN45 cells, 250 nM SN-38 treatment induces apoptosis by reducing pAkt expression but MKN1 cells showed resistance for apoptosis by no change in pAkt expression.

Therefore, inactivation of pAkt using the PI3K inhibitor, LY294002, finally induced apoptosis in MKN1 cells as well as in only SN-38-treated MKN45 cells. It was also reported (Nakashio et al., 2000) that constitutively active Akt results in a reduction of the cytotoxic effect of topotecan in lung carcinoma cells. The PI3K-Akt pathway might thus play a significant role in SN-38-induced apoptosis in human gastric cancer cells.

The tumor suppression gene PTEN is a natural biological inhibitor of Akt cell survival signals. PTEN expression sensitizes cells to death receptor-mediated apoptosis induced by a variety of stimuli, such as tumor necrosis factor (Mayo et al., 2002), TRAIL (Thakkar et al., 2001) and chemotherapeutic agents (Tanaka et al., 2000). Akt is abnormally activated in several human tumors by loss of PTEN (Di Cristofano and Pandolfi, 2000; Simpson and Parsons, 2001). Recent studies have documented that PTEN appears to negatively regulate PI3K-Akt survival signaling by removing the D-3 phosphate from PIP_3, which is a primary activator of Akt (Maehata and Dixon, 1998). Loss of PTEN results in high basal activity of Akt in a variety of tumors, while introduction of PTEN suppresses the activity. It is reported (Saga et al., 2002) that overexpression of PTEN increased sensitivity to SN-38 in ovarian cancer cells. However, in our study, the expression of PTEN was not correlated with SN-38-induced apoptosis; in human gastric cancer cells, SN-38 might suppress the PI3K-Akt signaling via a PTEN independent pathway. Rokudai et al. (2002) reported that suppression of PDK1, but not of PI3K, was one of the mechanisms of chemotherapeutic drug-mediated Akt inactivation, because VP-16 indirectly suppressed PDK1 kinase activity in 293T cells. However, the mechanisms of SN-38-mediated Akt inactivation remain unclear. Overexpression of constitutive active Akt using a transfected active form of *akt* cDNA overcame chemotherapeutic drug-induced apoptosis. Akt inactivation might be a sign of chemotherapeutic drug sensitivity of cancer cells, because it was found only in chemosensitive cells (MKN45).

Nuclear factor-κB (NF-κB) is a ubiquitously expressed transcription factor involved in a wide spectrum of cellular functions like cell cycle control, stress adaptation, inflammation and control of apoptosis (Pahl, 1999). NF-κB is constitutively activated in various types of tumors, including gastric cancer (Sasaki et al., 2001). Several investigators have reported that inhibition of the NF-κB pathway by adenoviral delivery of an IκBα superrepressor, small interfering RNA, against the p65 subunit of NF-κB or TNFα increased SN-38-induced apoptosis (Camp et al., 2004; Guo et al., 2004; Valente et al., 2003). Besides the PI3K-Akt pathway, the NF-κB pathway might also facilitate an antiapoptotic signal to inhibit SN-38-induced apoptosis.

In conclusion, we found that SN-38 induced apoptosis by decreasing the PI3K-Akt survival signaling, the antiapoptotic signals in human gastric cancer cells. A selective small molecule inhibitor of Akt, the Akt/protein kinase B signaling inhibitor-2 (API-2) was recently discovered by screening the National Cancer Institute Diversity Set (Yang et al., 2004). Such an Akt inhibitor might be a useful

anti-tumor agent in combination with a chemotherapeutic agent, such as CPT-11.

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References

1 Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, et al. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol 1997;7:261–269.

2 Balendran A, Casamayor A, Deak M, Paterson A, Gaffney P, Currie R, et al. PDK1 acquires PDK2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2. Curr Biol 1999;9:393–404.

3 Brognard J, Clark AS, Ni Y, Dennis PA. Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. Cancer Res 2001;61:3986–3997.

4 Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 1999;96:857–868.

5 Camp ER, Li J, Minnich DJ, Brank A, Moldawer LL, MacKay SL, et al. Inducible nuclear factor-kappaB activation contributes to chemotherapy resistance in gastric cancer. J Am Coll Surg. 2004;199: 249–258.

6 Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, et al. Regulation of cell death protease caspase-9 by phosphorylation. Science -1998;282:1318–1321.

7 Caserini C, Pratesi G, Tortoreto M, Bedogne B, Carenini N, Supino R, et al. Apoptosis as a determinant of tumor sensitivity to topotecan in human ovarian tumors: preclinical in vitro/in vivo studies. Clin Cancer Res 1997;3:955–961.

8 Chen Z, Naito M, Mashima T, Tsuruo T. Activation of actin-cleavable interleukin 1beta-converting enzyme (ICE) family protease CPP-32 during chemotherapeutic agent-induced apoptosis in ovarian carcinoma cells. Cancer Res 1996;56:5224–5229.

9 Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 1997;91:231–241.

10 Di Cristofano A, Pandolfi PP. The multiple roles of PTEN in tumor suppression. Cell 2000;100:387–390.

11 Editorial Board of the Cancer Statistics in Japan, The. Cancer statistics in Japan 2003. Tokyo: Foundation for Promotion of Cancer Research; 2003.

12 Falk SJ, Smith PJ. DNA damaging and cell cycle effects of the topoisomerase I poison camptothecin in irradiated human cells. Int J Radiat Biol 1992;61:749–757.

13 Franke TF, Kaplan DR, Cantley LC. PI3K: downstream AKTion blocks apoptosis. Cell 1997;88:435–437.

14 Guo J, Verma UN, Gaynor RB, Frenkel EP, Becerra CR. Enhanced chemosensitivity to irinotecan by RNA interference-mediated down-regulation of the nuclear factor-kappaB p65 subunit. Clin Cancer Res 2004;10:3333–3341.

15 Hemmings BA. Akt signaling: linking membrane events to life and death decisions. Science 1997;275:628–630.

16 Hickman JA. Apoptosis induced by anticancer drugs. Cancer Metastasis Rev 1992;11:121–139.

17 Kataoka S, Naito M, Tomida A, Tsuruo T. Resistance to antitumor agentinduced apoptosis in a mutant of human myeloid leukemia U937 cells. Exp Cell Res 1994;215:199–205.

18 Kawato Y, Aonuma M, Hirota Y, Kuga H, Sato K. Intracelular roles of SN-38, a metabolite of the camptothecin derivative CPT-11, in the antitumor effect of CPT-11. Cancer Res 1991;51:4187–4191.

19 Kerr JF, Winterford CM, Harmon BV. Apoptosis. Its significance in cancer and cancer therapy. Cancer 1994;73:2013–2026.

20 Kulik G, Klippel A, Weber MJ. Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. Mol Cell Biol 1997;17:1595–1606.

21 Kulik G, Weber MJ. Akt-dependent and -independent survival signaling pathways utilized by insulin-like growth factor I. Mol Cell Biol 1998;18:6711–6718.

22 Le Good JA, Ziegler WH, Parekh DB, Alessi DR, Cohen P, Parker PJ. Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. Science 1998;281:2042–2045.

23 Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem 1998;273:13375–13378.

24 Mayo MW, Madrid LV, Westerheide SD, Jones DR, Yuan XJ, Baldwin AS Jr, et al. PTEN blocks tumor necrosis factorinduced NF-kappa B-dependent transcription by inhibiting the transactivation potential of the p65 subunit. J Biol Chem 2002;277:11116–11125.
25 Nakashio A, Fujita N, Rokudai S, Sato S, Tsuruo T. Prevention of phosphatidylinositol 3'-kinase-Akt survival signaling pathway during topotecan-induced apoptosis. Cancer Res 2000;60:5303–5309.
26 Nam SY, Jung GA, Hur GC, Chung HY, Kim WH, Seol DW, et al. Upregulation of FLIP(S) by Akt, a possible inhibition mechanism of TRAIL-induced apoptosis in human gastric cancers. Cancer Sci 2003;94:1066–1073.
27 Ng SSW, Tsao MS, Chow S, Hedley DW. Inhibition of phosphatidylinositide 3-kinase enhances gemcitabine-induced apoptosis in human pancreatic cancer cells. Cancer Res 2000;60:5451–5455.
28 Osaki M, Kase S, Adachi K, Takeda A, Hashimoto K, Ito H. Inhibition of the PI3K-Akt signaling pathway enhances the sensitivity of Fas-mediated apoptosis in human gastric carcinoma cell line, MKN45. J Cancer Res Clin Oncol 2004;130:8–14.
29 Page C, Huang M, Jin X, Cho K, Lilja J, Reynolds RK, et al. Elevated phosphorylation of AKT and Stat3 in prostate, breast, and cervical cancer cells. Int J Oncol 2000;17:23–28.
30 Pahl HL. Activators and target genes of Rel/ NF-kappaB transcription factors. Oncogene 1999;18: 6853–6866.
31 Rokudai S, Fujita S, Kitahara O, Nakamura Y, Tsuruo T. Involvement of FKHR-dependent TRADD expression in chemotherapeutic drug-induced apoptosis. Mol Cell Biol 2002;22:8695–8708.
32 Romashkova JA, Makarov SS. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. Nature 1999;401:86–90
33 Saga Y, Mizukami H, Suzuki M, Kohno T, Urabe M, Ozawa K, et al. Overexpression of PTEN increases sensitivity to SN-38, an active metabolite of the topoisomerase I inhibitor irinotecan, in ovarian cancer cells. Clin Cancer Res 2002;8:1248–1252.
34 Sasaki N, Morisaki T, Hashizume K, Yao T, Tsuneyoshi M, Noshiro H, et al. Nuclear factor- kappaB p65 (RelA) transcription factor is constitutively activated in human gastric carcinoma tissue. Clin Cancer Res 2001;7:4136–4142.
35 Seimiya H, Mashima T, Toho M, Tsuruo T. c-Jun NH2-terminal kinase-mediated activation of interleukin-1beta-converting enzyme/CED-3-like protease during anticancer drug-induced apoptosis. J Biol Chem 1997;272:4631–4636.
36 Simpson L, Parsons R. PTEN: life as a tumor suppressor. Exp Cell Res 2001;264:29–41.
37 Stephens L, Anderson K, Stokoe D, Erdjument-Bromage H, Painter GF, Holmes A et al. Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. Science 1998;279:710–714.
38 Tanaka M, Koul D, Davies MA, Liebert M, Steck PA, Grossman HB. MMAC1/PTEN inhibits cell growth and induces chemosensitivity to doxorubicin in human bladder cancer cells. Oncogene 2000;19:5406–5412.
39 Thakkar H, Chen X, Tyan F, Gim S, Robinson H, Lee C, et al. Pro-survival function of Akt/protein kinase B in prostate cancer cells. Relationship with TRAIL resistance. J Biol Chem 2001;276:38361–38369.
40 Valente P, Arzani D, Cesario A, Margaritora S, Carbone E, Russo P. TNF increases camptothecin-induced apoptosis by inhibition of NF-kappaB. Eur J Cancer. 2003;39:1468–1477.
41 Yang L, Dan HC, Sun M, Liu Q, Sun XM, Feldman RI, et al. Akt/protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt. Cancer Res 2004;64:4394–4399.
42 Yoshida A, Ueda T, Wano Y, Nakamura T. DNA damage and cell killing by camptothecin and its derivative in human leukemia HL-60 cells. Jpn J Cancer Res 1993;84:566–573.
43 Yuan ZQ, Sun M, Feldman RI, Wang G, Ma X, Jiang C, et al. Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. Oncogene 2000;19:2324–2330,.
44 Xu Y, Villalona-Calero MA. Irinotecan: mechanism of tumor resistance and novel strategies for modulating its activity. Ann Oncol 2002;13:1841–1851.

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