SV40 T/t-Common Polypeptide Specifically Induces Apoptosis in Human Cancer Cells that Overexpress HER2/neu

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Abstract

Previously, we reported that SV40 T/t-common polypeptide, which contains the NH2-terminal common domain of SV40 large T and small t antigens, can repress HER2/neu (also known as erbB-2) expression and consequently suppress the tumorigenic potential of the HER2/neu-overexpressing ovarian carcinoma cells. Here we report that T/t-common could specifically induce apoptosis in HER2/neu-overexpressing human cancer cell lines but not in nontransformed cell lines and HER2/neu low-expressing human cancer cell lines. The ability of T/t-common to induce apoptosis in HER2/neu-overexpressing cancer cells was derived from its ability to inhibit HER2/neu because reexpression of a large amount of HER2/neu could block apoptosis induced by T/t-common. T/t-common expression in HER2/neu-overexpressing SK-OV-3 cancer cells led to down-regulation of Bcl-2 and Bcl-XL, and overexpression of Bcl-2 could inhibit the ability of T/t-common to induce apoptosis in these cells. Therefore, the apoptosis-inducing activity of T/t-common is related to its ability to inhibit Bcl-2 expression in HER2/neu-overexpressing cancer cells. Consistent with the apoptosis-inducing activity of T/t-common, we found that T/t-common could specifically inhibit the soft-agarose colony-forming ability of the HER2/neu-overexpressing human cancer cell lines but not that of the HER2/neu low-expressing human cancer cell lines. Finally, we showed that T/t-common could specifically sensitize HER2/neu-overexpressing human cancer cell lines, but not HER2/neu low-expressing human cancer cell lines, to chemotherapeutic agent etoposide. Together, these data suggest that T/t-common alone or in combination with chemotherapy may provide a new approach for treatment of cancers that overexpress HER2/neu. (Cancer Res 2006; 66(11): 5847-57)

Introduction

The HER2/neu (also known as erbB-2) proto-oncogene product is a member of the HER family of transmembrane receptor tyrosine kinases, which also includes the epidermal growth factor receptor (EGFR or HER1), HER3, and HER4 (1, 2). HER2/neu is a unique member of the HER family in that it does not bind any of the known ligands with high affinity but it is the preferred heterodimeric partner for other HER receptors (2–4). Through heterodimerization with other HER receptors, HER2/neu provides potent mitogenic, antiapoptotic, metastatic, and angiogenic signals (1–3). Amplification or overexpression of HER2/neu has been frequently detected in many types of human cancers, most notably breast and ovarian cancers in which ~30% overexpress HER2/neu (5–7). HER2/neu overexpression can lead to the formation of HER2/neu:HER2/neu homodimer, which may transduce oncogenic signal in the absence of specific ligands (1, 8). Alternatively, overexpressed HER2/neu may promote tumor formation as a result of spontaneous or ligand-induced heterodimerization with other HER receptors (2, 9). HER2/neu overexpression has been shown to enhance proliferative, prosurvival, and metastatic signals in breast cancer cells (1, 2, 10). Preclinical data show that HER2/neu activation contributes more to the oncogenic phenotype than any of the other HER receptors (3, 11). In fact, overexpression of HER2/neu has been shown to be correlated with poor prognosis (5, 7, 10, 12), enhanced metastatic potential (7, 12, 13), and increased chemoresistance (7, 10, 14) of human cancers. Taken together, these results indicate that HER2/neu is a potent oncogene and its overexpression plays an important role in the development of human malignancies. These results also imply that by repressing HER2/neu overexpression, it is possible to achieve therapeutic effects on cancers that overexpress HER2/neu.

A number of attempts have been made to inhibit HER2/neu expression or activity in cancer cells as a potential therapeutic approach; some have shown great promise in this regard (reviewed in refs. 10, 15, 16). Most notably, a humanized monoclonal antibody against HER2/neu (Trastuzumab or Herceptin) has been shown to be effective in suppressing HER2/neu-overexpressing metastatic cancers (16). Two viral proteins, adenovirus E1A and SV40 large T, have been reported to repress HER2/neu expression and consequently suppress the tumorigenicity of HER2/neu-overexpressing cancer cells (17, 18). Through inhibition of HER2/neu expression, adenovirus E1A not only can inhibit HER2/neu-overexpressing tumor cell growth in vitro but also can suppress the tumorigenic potential of breast and ovarian cancers that overexpress HER2/neu in vivo (10, 19). Moreover, E1A can effectively inhibit metastasis of HER2/neu-overexpressing tumor cells and sensitize those cells to chemotherapeutic agents (10, 20). In view of their potent tumor-suppressing activity, both E1A and large T have been used in gene therapy experiments for treatment of HER2/neu-overexpressing cancers (21, 22). However, a serious concern exists for these approaches, as E1A and large T are potent viral oncoproteins which, either alone or in cooperation with a second oncoprotein, can transform primary cells (23, 24).

In search for other viral proteins that can inhibit HER2/neu expression, we previously reported that SV40 T/t-common polypeptide, which contains the NH2-terminal common domain

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of SV40 large T and small t antigens, can specifically repress HER2/neu expression in the HER2/neu-overexpressing ovarian carcinoma cells (25). We also reported that T/t-common, through inhibition of HER2/neu, can suppress the tumorigenic potential of the HER2/neu-overexpressing ovarian cancer cells (25). T/t-common has been shown to have strong transcriptional repressing activity (26) and express a biological activity equivalent to the NH2-terminal nonconserved domain and the conserved domain 1 of E1A (27). Although T/t-common contains a dnal domain, which has been shown to be required for large T transformation of cells (23), it alone lacks transforming activity (25, 28). Taken together, these data suggest that T/t-common may be safer than E1A and large T for the treatment of HER2/neu-overexpressing cancers.

To further investigate the tumor-suppressing activity of T/t-common on HER2/neu-overexpressing cancer cells, in this study, we investigated the apoptosis-inducing ability of T/t-common. Here we report that T/t-common could specifically induce apoptosis in tumor cell lines that overexpress HER2/neu but not in nontransformed cells and tumor cell lines that express low levels of HER2/neu. We also found that T/t-common could specifically suppress the tumorigenic potential of the HER2/neu-overexpressing cancer cells and specifically sensitize HER2/neu-overexpressing cancer cells to chemotherapeutic agent etoposide. The ability of T/t-common to accomplish selective cytotoxicity against HER2/neu-overexpressing cells to chemotherapeutic agent etoposide, the potential of T/t-common to suppress the tumorigenic potential of the HER2/neu-overexpressing ovarian carcinoma cells (25). T/t-common has been shown to have strong transcriptional repressing activity (26) and express a biological activity equivalent to the NH2-terminal nonconserved domain and the conserved domain 1 of E1A (27).

Adenoviral constructs. The recombinant adenovirus expressing the T/t-common polypeptide, rAd-T/t-common, was generated using the AdEasy adenoviral vector system (kindly provided by Bert Vogelstein at Howard Hughes Institute, Johns Hopkins University, Baltimore, MD; ref. 32). Briefly, the HindIII-XbaI fragment with (XbaI site blunted) of pRSV-T/t-common, which contains the T/t-common coding sequence, was inserted into the HindIII/EcoRV sites of the adenoviral shuttle vector pAdTrack-CMV, which contains a GFP marker gene under the control of a separate CMV-IE promoter, to generate pAdTrack-CMV-T/t-common. The pAdTrack-CMV-T/t-common, which contains the T/t-common coding sequence, was linearized with the restriction enzyme PmeI, and then cotransformed with pAdEasy-1, containing the adenoviral genome, into Escherichia coli strain BJ5183, where homologous recombination took place. Positive clones were identified by restriction analysis. The resultant recombinant pAdEasy-1-T/t-common, which can express the T/t-common polypeptide, was linearized with restriction enzyme PacI and transfected into a packaging cell line (293 cells) to produce the recombinant adenovirus (rAd-T/t-common) expressing the T/t-common polypeptide. The control vector adenovirus expressing only the GFP transgene, rAd-V, was prepared in a similar way except that pAdTrack-CMV was used in the recombination.

Establishment of HER2/neu-reexpressing stable transfectants. SK-OV-3-T/t-3 and SK-OV-3-T/t-4 cells were transfected with 11 μg of either pcDNA3 or pCMVHER2WT together with 1 μg of pCEP4-dlEBNA1, which contains a hygromycin resistance gene, by calcium phosphate coprecipitation method as previously described (25). The cells were trypsinized and then split at a 1:10 ratio 48 hours after transfection. After 4 to 6 weeks of selection, medium containing 200 μg/mL of hygromycin (Calbiochem, La Jolla, CA), individual hygromycin-resistant colonies were cloned and tested for the expression of HER2/neu by Western blot analysis.

Flow cytometric detection of apoptosis. To assess apoptosis, both floating and attached cells were collected, combined together, washed thrice with PBS, and fixed in 70% ice-cold ethanol for 2 hours. Fixed cells were washed twice with PBS and incubated in 1 mL of PBS containing 50 μg/mL propidium iodide (Sigma, St. Louis, MO) and 200 μg/mL of heat-treated RNase (Sigma) at 37°C for 30 minutes. Stained cells were then analyzed on a FACSCalibur Flow Cytometer (Becton Dickinson, Mountain View, CA) using CELLQuest software (Becton Dickinson). Cells undergoing apoptosis were determined as a percentage of cells with sub-G1 DNA content in the DNA histogram compared with the total number of cells analyzed.

Western blot analysis, terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling assay, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining. Western blot analyses were done as previously described (33). Equivalent amounts of extract protein were used in the analysis. The terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was done using the in situ cell death detection kit (Roche Applied Science, Mannheim, Germany). The coverslip containing labeled cells was subjected to fluorescence microscopy examination using Zeiss Axiophot upright microscope (Zeiss, Jena, Germany). For 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining, cells expressing β-galactosidase were histochemically stained with the substrate X-gal as previously described (19).

Antibodies. Mouse monoclonal antibody against HER2/neu (c-neu-Ab-3) was purchased from Oncogene Science (Cambridge, MA). Mouse monoclonal antibodies against phospho-extracellular signal-regulated kinase (ERK)-1/2, Bcl-2, and Bax, mitogen-activated protein kinase/ERK kinase (MEK)-1, and goat polyclonal antibody against β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal antibodies against Bcl-XL, ERK1, and ERK2 were purchased from BD Transduction Labs, BD Pharmingen (San Diego, CA). Mouse monoclonal antibody against α-tubulin was purchased from NeoMarkers, Inc. (Freemont, CA). Mouse monoclonal antibody against glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was purchased from Biodesign (Saco, ME). Rabbit polyclonal antibodies against phospho-MEK1/2, cleaved caspase-3, and cleaved poly(ADP-ribose)polymerase were purchased from Cell Signaling Technology (Beverly, MA).

G418-resistant colony formation assay. Cells were transfected with 11 μg of either pCMV-T/t-common or its vector plasmid pcDNA3.1, which
contains a neomycin resistance gene, by calcium phosphate coprecipitation method as previously described (25). One microgram of pEGFP-C1, which contains a CMV-IE-driven GFP gene, was also cotransfected as an internal control for transfection efficiency. The transfected cells were seeded in 100-mm dishes at appropriate cell densities and incubated with medium containing 0.5% FBS and appropriate concentration of G418 (Promega Corporation, Madison, WI). Three days later, the medium was removed and replaced with medium containing 10% FBS and appropriate concentration of G418. Cells were maintained in the latter medium for 18 days. Cells were then fixed and stained with 0.1% crystal violet in 20% ethanol. The G418-resistant colonies were counted and calibrated against transfection efficiency.

**Results**

**Induction of apoptosis by SV40 T/t-common polypeptide in HER2/neu-overexpressing human ovarian carcinoma SK-OV-3 cells.** Previously, we reported that SV40 T/t-common polypeptide can inhibit HER2/neu expression in HER2/neu-overexpressing ovarian carcinoma SK-OV-3 cells (25). Because inhibition of HER2/neu can lead to apoptosis of HER2/neu-overexpressing cancer cells (34–36), we therefore tested whether T/t-common could induce apoptosis in HER2/neu-overexpressing SK-OV-3 cells. To do this, three T/t-common–expressing SK-OV-3 clones (SK-T/t-1, SK-T/t-3, and SK-T/t-4) and one vector-transfected clone (SK-NEO-2) previously established in our laboratory (25) were used. The expression of T/t-common polypeptide in these SK-OV-3-derived clones is shown in Fig. 1A. These T/t-common–expressing SK-OV-3 clones contain copy number and structure of the HER2/neu gene similar to that of the vector-transfected clones and parental SK-OV-3 cells (25). However, they expressed much lower amount of HER2/neu protein than the vector-transfected SK-NEO-2 and parental SK-OV-3 cells (Fig. 1A), indicating that T/t-common can inhibit HER2/neu expression in SK-OV-3 cells. To investigate whether these T/t-common–expressing SK-OV-3 clones underwent apoptosis, the various SK-OV-3 clones were grown in the medium containing 10% FBS for 96 hours and then analyzed for DNA content by flow cytometry. As shown in Fig. 1B, the percentage of cells in sub-G1 phase was significantly higher in the T/t-common–expressing SK-OV-3 clones than in the vector-transfected and parental cells, indicating that T/t-common can induce apoptosis in SK-OV-3 cells. Because SK-OV-3 cells also express significant

![Image](99x176 to 484x461)

**Figure 1.** Induction of apoptosis of HER2/neu-overexpressing SK-OV-3 cancer cells by SV40 T/t-common polypeptide. A, expression of T/t-common and HER2/neu in T/t-common–transfected, vector-transfected, and parental SK-OV-3 cell lines. Top, Western blot analysis of the HER2/neu protein in various cell lines. α-Tubulin served as an internal control for amounts of protein loaded on the gel. Bottom, immunoprecipitation analysis of T/t-common protein in various cell lines. Cells were labeled and lysed, and the extracts were immunoprecipitated with anti-T/t-common monoclonal antibody pAB419 as previously described (26). B, sub-G1 DNA content analysis showing that T/t-common–expressing SK-OV-3 clones underwent apoptosis under high-serum condition. Columns, average of three independent experiments; bars, SD. *, P < 0.05; †, P < 0.005, comparison between parental SK-OV-3 cells and T/t-common–transfected cell lines (unpaired Student’s t test). C, sub-G1 DNA content analysis showing that T/t-common–expressing SK-OV-3 clones underwent apoptosis under low-serum condition. Cells were incubated in DMEM/F12 medium containing 0.1% FBS for 96 hours. Columns, average of two independent experiments; bars, SD. *, P < 0.02, comparison between vector-transfected SK-OV-3 cells and T/t-common–transfected cell lines (unpaired Student’s t test). D, Hoechst 33258 staining of SK-BR-3 cells transiently transfected with vector (top) or T/t-common–encoding (bottom) plasmid. SK-BR-3 cells seeded on poly-L-lysine–coated coverslips were transiently cotransfected with a GFP-encoding plasmid (pEGFP-F) together with either a T/t-common–encoding plasmid (pRSV-T/t-common) or a vector plasmid (pRSV-3-BglII). After transfection, cells were cultured in DMEM/F12 medium containing 0.5% FBS. Forty-eight hours later, cells were fixed and stained with Hoechst 33258 to label the nuclear DNA. Left, cells that express GFP; right, Hoechst 33258 staining of the cells in the same microscopic field (magnification, ×1,000).
amount of EGFR (37, 38), which may transduce proliferative and prosurvival signals in the presence of serum, we figured that T/t-common might be more potent in inducing apoptosis of SK-OV-3 cells in low-serum medium. We thus grew these T/t-common–expressing SK-OV-3 clones and vector-transfected SK-OV-3 clone in low-serum medium and subjected these cells to sub-G₁ DNA content analysis. T/t-common–expressing SK-OV-3 clones indeed underwent severe apoptosis in low-serum medium, in sharp contrast to the vector-transfected SK-OV-3 clone which had much lower percentage of cells in sub-G₁ phase (Fig. 1C). The TUNEL assay and Hoechst 33258 staining of cell nuclei also indicated that under low-serum condition, many of the cells from T/t-common–expressing SK-OV-3 clones underwent apoptosis (with fluorescent nuclei in TUNEL assay and condensed nuclei in Hoechst staining) whereas vector-transfected SK-OV-3 cells did not (Supplementary Fig. S1). Because T/t-common seems to induce apoptosis more efficiently in low-serum medium (compare Fig. 1B and C), most of the following experiments on T/t-common induction of apoptosis were done under low-serum condition.

To further confirm that T/t-common could induce apoptosis in HER2/neu-overexpressing cancer cells, we transiently cotransfected either T/t-common–expressing or vector plasmid together with GFP-expressing plasmid into HER2/neu-overexpressing SK-BR-3 cancer cells, which exhibited much better transfection efficiency than SK-OV-3 cells. We then examined the nuclear morphology of the GFP-expressing cells using Hoechst 33258 staining of the nuclear DNA. The vast majority of the GFP-expressing cells in the vector-cotransfected group had intact nuclear morphology as visualized by Hoechst 33258 staining (Fig. 1D, top). In striking contrast, many of the GFP-expressing cells in the T/t-common–cotransfected group displayed typical features of apoptotic nuclei, such as chromatin condensation or nuclear fragmentation (Fig. 1D, bottom). These data further confirm that T/t-common can induce apoptosis in HER2/neu-overexpressing cancer cells.

The ability of T/t-common to induce apoptosis in HER2/neu-overexpressing cancer cells was derived from its ability to repress HER2/neu expression. Previous studies have shown that repression of HER2/neu expression in HER2/neu-overexpressing cancer cells can lead to apoptosis (34–36). Because T/t-common can repress HER2/neu expression in HER2/neu-overexpressing SK-OV-3 cancer cells (Fig. 1A; ref. 25), we suspected that the ability of T/t-common to induce apoptosis in SK-OV-3 cells might be derived from its ability to inhibit HER2/neu expression. To investigate whether this hypothesis was true, we tested whether reexpression of HER2/neu in T/t-common–expressing SK-OV-3 cells could block apoptosis induced by T/t-common. We reintroduced the HER2/neu gene under the control of CMV-IE promoter (which is not repressed by T/t-common) into the T/t-common–expressing SK-OV-3 clone, SK-T/t-4, to generate two HER2/neu-reexpressing clones, SK-T/t-4-H1 and SK-T/t-4-H2. Both SK-T/t-4-H1 and SK-T/t-4-H2 clones produced a large amount of HER2/neu whereas the vector-transfected clone, SK-T/t-4-V1, produced similar amount of HER2/neu as the parental clone (Fig. 2A, top). TUNEL staining of these clones showed that whereas the parental and vector-transfected clones contained similar levels of apoptotic cells, the HER2/neu-reexpressing clones contained much reduced levels of apoptotic cells (Fig. 2A, bottom). These data indicate that reexpression of HER2/neu can block the ability of T/t-common to induce apoptosis in HER2/neu-overexpressing SK-OV-3 cells. A similar conclusion was obtained when HER2/neu was reexpressed in another T/t-common–expressing SK-OV-3 clone, SK-T/t-1-3 (Fig. 2B).

To rule out the possibility that the above observations were due to clonal selection of nonapoptotic clones, we also did transient cotransfection assays. We cotransfected pCMV-β, which contains the β-galactosidase-encoding gene driven by the CMV-IE promoter, with either the T/t-common–encoding plasmid alone or the T/t-common–encoding plasmid plus the CMV-IE-driven HER2/neu-expressing plasmid into HER2/neu-overexpressing breast cancer cells, AU565, which exhibited much better transfection efficiency than SK-OV-3. As shown in Fig. 2C, T/t-common cotransfection led to reduced number of blue cells as compared with vector cotransfection (compare column 1 with column 2), indicating that T/t-common causes the death of the transfected cells. When HER2/neu was cotransfected with T/t-common, the number of blue cells increased significantly as compared with T/t-common transfection alone (compare column 2 with column 3). These data indicate that cotransfection of HER2/neu can block the ability of T/t-common to kill AU565 cells. Taken together, the above data show that reexpression of HER2/neu can block the ability of T/t-common to induce apoptosis in HER2/neu-overexpressing cancer cells. Because T/t-common is known to be able to inhibit HER2/neu expression (25), these data suggest that the ability of T/t-common to induce apoptosis of HER2/neu-overexpressing cancer cells is derived from its ability to inhibit HER2/neu expression. To further confirm this, we also tested whether T/t-common could inhibit the expression of other HERs in HER2/neu-overexpressing cancer cells. The result was negative (Fig. 2D).

Modulation of apoptosis regulatory molecules by T/t-common in HER2/neu-overexpressing SK-OV-3 cells. Previous studies have shown that overexpression of HER2/neu can lead to up-regulation of antiapoptotic Bcl-2 and Bcl-X₁ proteins (39, 40) and that inhibition of HER2/neu in HER2/neu-overexpressing cancer cells can result in apoptosis and down-regulation of Bcl-2 and Bcl-X₁ (40, 41). Because T/t-common could inhibit HER2/neu expression and induce apoptosis in HER2/neu-overexpressing SK-OV-3 cells, we tested whether T/t-common could down-regulate the expression of Bcl-2 and Bcl-X₁ in these cells. To do this, the expression level of Bcl-2, Bcl-X₁, and Bax was compared in vector-transfected SK-OV-3 clone and T/t-common–expressing SK-OV-3 clones. As shown in Fig. 3A, whereas all of the SK-OV-3-derived clones expressed similar level of Bax protein, the T/t-common–expressing SK-OV-3 clones produced lower amount of Bcl-2 and Bcl-X₁ than the vector-transfected SK-OV-3 clone. These data suggest that T/t-common can inhibit the expression of Bcl-2 and Bcl-X₁ in SK-OV-3 cells and this inhibition may contribute to the ability of T/t-common to induce apoptosis in HER2/neu-overexpressing cancer cells.

To confirm that the ability of T/t-common to induce apoptosis in HER2/neu-overexpressing cancer cells was at least partially mediated by its ability to inhibit Bcl-2 expression, we tested whether introduction of Bcl-2 could block the ability of T/t-common to kill HER2/neu-overexpressing AU565 cancer cells by transient β-galactosidase gene cotransfection assay as described above. Indeed, cotransfection of Bcl-2-expressing plasmid could inhibit the cytotoxic effect of T/t-common on HER2/neu-overexpressing AU565 cancer cells (Fig. 3B), suggesting that T/t-common induction of apoptosis of HER2/neu-overexpressing cancer cells is mediated through down-regulation of Bcl-2 in these cells.

HER2/neu overexpression is known to activate several prosurvival signaling pathways, one of them is the MEK-ERK pathway (39).
Activation of the MEK-ERK pathway has been shown to be able to up-regulate the expression of Bcl-2 and Bcl-XL (39, 42). Because T/t-common can inhibit HER2/neu expression, we suspected that T/t-common might be able to inhibit the MEK-ERK pathway and this inhibition might then result in Bcl-2 and Bcl-XL down-regulation. To test this hypothesis, we measured the activities of MEK and ERK in T/t-common–expressing and vector-transfected SK-OV-3 clones by using antibodies against phospho-MEK1/2 and phospho-ERK1/2, respectively. Phosphorylation of MEK1/2 and ERK1/2 at specific threonine and serine residues is known to activate the kinases, and antibodies to the phosphorylated form can be used to monitor the activity of these enzymes in the cell lysates. Compared with the vector-transfected SK-OV-3 clone, the activity of MEK1/2 and ERK1/2 was significantly reduced in the T/t-common–expressing SK-OV-3 clones as measured by the phospho-MEK1/2- and phospho-ERK1/2-specific antibodies (Fig. 3C and D). In contrast, the overall level of MEK1 and ERK1/2 proteins was similar in all of the SK-OV-3-derived clones. Moreover, rAd-T/t infection also led to the down-regulation of MEK activity but not MEK expression in HER2/neu-overexpressing cancer cells (Supplementary Fig. S2). Therefore, T/t-common inhibited the activity but not the expression of MEK1 and ERK1/2 in HER2/neu-overexpressing cancer cells. In vitro MEK kinase assay further confirms that the kinase activity of MEK1 is inhibited in T/t-common–expressing SK-OV-3 clones (Fig. 3E). That inhibition of MEK activity may contribute to the ability of T/t-common to induce apoptosis in HER2/neu-overexpressing cancer cells.
cells was shown by transient β-galactosidase gene cotransfection assay as described above. As shown in Fig. 3F, cotransfection of constitutively active MEK could block the killing effect of T/t-common on HER2/neu-overexpressing cancer cells.

**T/t-common could induce apoptosis in HER2/neu-overexpressing human cancer cell lines but not in nontransformed cell lines and HER2/neu low-expressing human cancer cell lines.** To test whether T/t-common could induce apoptosis in other HER2/neu-overexpressing cancer cell lines, a panel of HER2/neu-overexpressing human cancer cell lines, including breast cancer cell lines, MDA-MB-453, SK-OV-3, SK-BR-3, and BT-474, and ovarian carcinoma cell lines, SK-OV-3.ip1 and SK-OV-3, were used. The expression levels of HER2/neu protein in these HER2/neu-overexpressing cancer cell lines, some HER2/neu low-expressing cancer cell lines, and nontransformed cell lines were shown in Fig. 4A. We first tested whether T/t-common could induce cell death in HER2/neu-overexpressing cancer cell lines by G418-resistant colony formation assays. We transfected either a T/t-common–encoding plasmid, pCMV-T/t-common, which also contains a neomycin resistance gene, or its vector control plasmid into HER2/neu-overexpressing human cancer cell lines (MDA-MB-453, SK-OV-3.ip1, SK-OV-3, and AU565), HER2/neu low-expressing human cancer cell lines (NPC-TW04, HeEp-2, HeLa, and MCF-7), or nontransformed cell lines (CV-1 and NIH-3T3). Following selection with G418, the number of G418-resistant colonies was counted. We found that T/t-common transfection led to much lower number of G418-resistant colonies than vector transfection in all of the HER2/neu-overexpressing cancer cell lines (Fig. 4B). These data suggest that T/t-common can either severely inhibit cell growth or induce cell death in these HER2/neu-overexpressing cancer cell lines. In contrast, in nontransformed cell lines and HER2/neu low-expressing cancer cell lines, transfection with T/t-common or vector plasmid led to similar number of G418-resistant colonies (Fig. 4C), suggesting that these cell lines are resistant to the inhibitory or killing effect of T/t-common.

We next investigated whether T/t-common could induce apoptosis in various HER2/neu-overexpressing cancer cell lines. HER2/neu-overexpressing human cancer cell lines (SK-BR-3, AU565, MDA-MB-453, SK-OV-3, SK-OV-3.ip1, and BT-474), HER2/neu low-expressing human cancer cell lines (MCF-7, MDA-MB-231, HeLa, NPC-TW04, and H1299), and nontransformed cell lines (CV-1 and MRC-5) were infected with either a recombinant adenovirus...
carrying the T/t-common gene, rAd-T/t, or a vector adenovirus, rAd-V. rAd-T/t infection of SK-OV-3 cells could produce a significant amount of T/t-common protein whereas rAd-V infection could not (Fig. 4D). After incubation in low-serum medium, the cells were subjected to sub-G₁ DNA content analysis. As shown in Fig. 4E, compared with rAd-V infection, rAd-T/t infection dramatically increased the amount of apoptotic cells (i.e., sub-G₁ cells) in all of the HER2/neu-overexpressing cancer cell lines. In contrast, in nontransformed cell lines and HER2/neu low-expressing cancer cell lines, the level of apoptotic cells was similar after either rAd-T/t or rAd-V infection. In addition, we also found that HER2/neu-overexpressing cancer cell lines infected by an unrelated recombinant adenovirus expressing IL-12 did not undergo apoptosis (Fig. 4F). Together, these data show that T/t-common

Figure 4. T/t-common could induce apoptosis in HER2/neu-overexpressing cancer cell lines but not in nontransformed cell lines and HER2/neu low-expressing cancer cell lines. A, Western blot analysis of the HER2/neu protein in cell lysates of HER2/neu-overexpressing cancer cell lines (SK-OV-3, AU565, SK-BR-3, MDA-MB-453, SK-OV-3.ip1, and BT-474), HER2/neu low-expressing cancer cell lines (MCF-7, HeLa, HeP-2, NPC-TW04, MDA-MB-231, and H1299), and nontransformed cell lines (MRC-5, NIH-3T3, and CV-1). B, T/t-common transfection could inhibit G418-resistant colony formation in HER2/neu-overexpressing cancer cell lines. The experiment was done as described in Materials and Methods. Columns, average of two independent experiments; bars, SD. *, P < 0.02, comparison between vector-transfected cells and T/t-common–transfected cells (unpaired Student’s t test). C, T/t-common transfection could not inhibit G418-resistant colony formation in HER2/neu low-expressing cancer cell lines and nontransformed cell lines. Columns, average of two independent experiments; bars, SD. D, immunoprecipitation of T/t-common protein following infection of SK-OV-3 cells with rAd-T/t. SK-OV-3 cells were infected with rAd-V or rAd-T/t at 150 MOI, or mock infected. The expression of T/t-common was detected by immunoprecipitation as described in Fig. 1A. Cell extracts from 293 cells transfected with T/t-common–expressing plasmid, pRSV-T/t-common, served as a positive control for the production of T/t-common (lane 1). E, rAd-T/t infection induced apoptosis in HER2/neu-overexpressing cancer cell lines but not in HER2/neu low-expressing cancer cell lines and nontransformed cell lines. For each cell line, cells were infected with either rAd-T/t or rAd-V at appropriate MOI, such that >90% of the cells were infected. The infection efficiency by rAd-V and rAd-T/t was similar in each cell line tested. The infected cells were then incubated in medium containing 0.5% FBS for various lengths of time (44 hours for SK-BR-3, AU565, and MDA-MB-453; 72 hours for SK-OV-3, SK-OV-3.ip1, and BT-474; 96 hours for MCF-7, MDA-MB-231, HeLa, NPC-TW04, H1299, CV-1, and MRC-5). Cells were then harvested and subjected to sub-G₁ DNA content analysis. The level of apoptosis induced by rAd-V infection was set at 1 and the level of apoptosis induced by rAd-T/t infection was expressed relative to this value. Columns, average of two independent experiments; bars, SD. *, P < 0.05, comparison between rAd-V-infected cells and rAd-T/t-infected cells (unpaired Student’s t test). F, rAd-IL-12 infection could not induce apoptosis in HER2/neu-overexpressing cancer cell lines. For each cell line, cells were infected with either rAd-V or rAd-IL-12 (kindly provided by Bor-Luen Chiang, National Taiwan University Hospital, Taipei, Taiwan) at same MOI. The level of apoptosis induced by rAd-V infection was set at 1 and the level of apoptosis induced by rAd-IL-12 infection was expressed relative to this value. Columns, average of two independent experiments; bars, SD.
can specifically induce apoptosis in HER2/neu-overexpressing cancer cell lines but not in nontransformed cell lines and HER2/neu low-expressing cancer cells. The TUNEL and Hoechst nuclei staining assays also showed that rAd-T/t infection could specifically induce apoptosis in HER2/neu-overexpressing cancer cells but not in nontransformed cells and HER2/neu low-expressing cancer cells (Supplementary Figs. S3 and S4).

To study whether T/t-common induced apoptosis of HER2/neu-overexpressing cancer cells through activation of caspase activity, we measured the caspase activity of rAd-T/t- or rAd-V-infected AU565 cells by detecting the cleavage product of poly(ADP-ribose)polymerase, which is the substrate of caspase-3 and other caspases. As shown in Fig. 5A, rAd-D/T infection caused a significant increase in the cleavage product of poly(ADP-ribose)polymerase than rAd-V infection, indicating that T/t-common expression can lead to activation of caspase activity in HER2/neu-overexpressing AU565 cells. We next examined whether procaspase-3 was activated after rAd-D/T infection of AU565 cells. Compared with rAd-V infection, the cleavage of procaspase-3 was significantly increased after rAd-D/T infection (Fig. 5B), suggesting that caspase-3 is activated by T/t-common in HER2/neu-overexpressing AU565 cancer cells.

T/t-common could specifically suppress the in vitro transforming ability of the HER2/neu-overexpressing human cancer cell lines but not of the HER2/neu low-expressing human cancer cell lines. The above data suggest that T/t-common may be able to specifically suppress the tumorigenic potential of HER2/neu-overexpressing cancer cell lines. To test this possibility, HER2/neu-overexpressing human cancer cell lines (SK-OV-3, SK-OV-3.ip1, AU565, MDA-MB-453, and BT-474) and HER2/neu low-expressing human cancer cell lines (MDA-MB-231, NPC-TW04, HeLa, MCF-7, and HEP2) were infected with either rAd-V or rAd-D/T, seeded in soft agarose containing 10% FBS, and the number of colonies formed was counted at 28 days postseeding. Compared with rAd-V infection, rAd-D/T infection could dramatically suppress the soft-agarose colony-forming ability of the HER2/neu-overexpressing cancer cell lines (Fig. 6A) but not that of the HER2/neu low-expressing cancer cell lines (Fig. 6B). An unrelated recombinant adenovirus expressing IL-12 was also tested for its ability to suppress the soft-agarose colony-forming ability of the HER2/neu-overexpressing cancer cell lines (Fig. 6C). The result was negative. Together, these data clearly indicate that T/t-common can specifically suppress the in vitro transforming ability of the HER2/neu-overexpressing cancer cells but not that of the HER2/neu low-expressing cancer cells. The number of colonies formed by the rAd-D/T-infected SK-OV-3 and AU565 cancer cells remained very low even at 70 days postseeding (data not shown). This result indicates that rAd-D/T infection induces cell death rather than suppressing cell growth in these HER2/neu-overexpressing cancer cells because the expression of the genes carried by adenovirus is transient. T/t-common can suppress the tumorigenicity of HER2/neu-overexpressing cancer cells was also supported by our preliminary data which showed that rAd-D/T infection could reduce SK-OV-3 tumor size in nude mice model (data not shown).

T/t-common could specifically enhance etoposide-induced apoptosis in HER2/neu-overexpressing human cancer cell lines but not in HER2/neu low-expressing human cancer cell lines. Previous studies have shown that HER2/neu overexpression can confer resistance to chemotherapeutic agents in various cancer cell lines (7, 14, 43) and that inhibition of HER2/neu can sensitize HER2/neu-overexpressing cancer cells to chemotherapeutic agents (44, 45). Because T/t-common can inhibit HER2/neu, we thus tested whether T/t-common could sensitize HER2/neu-overexpressing cancer cells to the chemotherapeutic agent etoposide, a topoisomerase II inhibitor. The HER2/neu-overexpressing human cancer cell lines (BT-474, SK-OV-3, SK-OV-3.ip1, and SK-BR-3) or the HER2/neu low-expressing human cancer cell lines (NPC-TW04, HeLa, and HEP2) were treated with rAd-V alone, rAd-D/T alone, etoposide alone, rAd-V plus etoposide, or rAd-D/T plus etoposide. After incubation in media containing 10% FBS, the cells were harvested and subjected to sub-G1 DNA content analysis. As shown in Fig. 7A, rAd-D/T infection could significantly enhance etoposide-induced apoptosis, whereas rAd-V infection could not, in all the HER2/neu-overexpressing cancer cell lines tested. By contrast, in HER2/neu low-expressing cancer cell lines, neither rAd-D/T nor rAd-V infection increased the rate of apoptosis induced by etoposide (Fig. 7B). Taken together, the above data show that T/t-common can specifically enhance apoptosis induced by etoposide in HER2/neu-overexpressing cancer cells but not in HER2/neu low-expressing cancer cells.

**Discussion**

In recent years, the HER2/neu oncogene has received a great deal of attention as a potential therapeutic target in human cancers. HER2/neu is an attractive target for molecular therapeutic both because it is overexpressed in a significant fraction of human cancers and because it plays a critical role in the development of tumors that overexpress HER2/neu. Interference with HER2/neu expression or function can inhibit the neoplastic growth of HER2/neu-overexpressing tumor cells and often lead to apoptosis of these tumor cells (10, 15, 16, 34, 35, 36). Previously, we reported that SV40 T/t-common polypeptide can inhibit HER2/neu expression and

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**Figure 5.** rAd-D/T infection stimulated the activity of caspases in HER2/neu-overexpressing AU565 cancer cells. A, caspase substrate poly(ADP-ribose)polymerase (PARP) was cleaved in AU565 cells after rAd-D/T infection. AU565 cells were infected with rAd-D/T, rAd-V (at 50 MOI), or mock infected. After infection, cells were cultured in DMEM medium containing 0.5% FBS for 72 hours. Cell lysates were then prepared and immunoblotted with antibodies against cleaved poly(ADP-ribose)polymerase fragment. B, the activity of caspase-3 was activated in AU565 cells after rAd-D/T infection. AU565 cells were infected with rAd-D/T, rAd-V (at 50 MOI), or mock infected. After infection, cells were cultured in DMEM medium containing 0.5% FBS for 72 or 96 hours. Cell lysates were then prepared and immunoblotted with antibodies against cleaved caspase-3 fragment.
consequently suppress the tumorigenic potential of the HER2/neu-overexpressing SK-OV-3 ovarian cancer cells (25). In this study, we further showed that T/t-common could specifically induce apoptosis and suppress the tumorigenicity of various HER2/neu-overexpressing human cancer cell lines. More importantly, we also found that T/t-common could specifically enhance drug-induced apoptosis in HER2/neu-overexpressing human cancer cell lines but not in HER2/neu low-expressing human cancer cell lines. These data suggest that T/t-common gene therapy may improve the therapeutic efficacy of chemotherapeutic agents in the treatment of HER2/neu-overexpressing tumors.

Several of the HER2/neu-overexpressing cancer cell lines studied here, including SK-OV-3 and SK-BR-3, have known p53 mutations (36, 46). Thus, the effect of T/t-common in activating apoptosis in HER2/neu-overexpressing cancer cells must be p53 independent. T/t-common could inhibit HER2/neu (25) but could not affect the expression of EGFR and HER3 in HER2/neu-overexpressing cancer cells (Fig. 2D). Thus, the ability of T/t-common to induce apoptosis in HER2/neu-overexpressing cancer cells might be derived from its ability to inhibit HER2/neu. We found that this is indeed the case because reexpression of a large amount of HER2/neu could block the ability of T/t-common to induce apoptosis in HER2/neu-overexpressing cancer cells (Fig. 2). We also showed that T/t-common could inhibit the activity of MEK1/2 and ERK1/2 (Fig. 3C-E and Supplementary Fig. S2) and the expression of Bcl-2 and Bcl-XL (Fig. 3A) in HER2/neu-overexpressing SK-OV-3 cancer cells. Because HER2/neu overexpression can lead to the activation of its downstream signaling proteins MEK and ERK (39), which in turn up-regulate the expression of Bcl-2 and Bcl-XL (39, 42), we propose the following model to illustrate the possible mechanism through which T/t-common specifically enhanced apoptosis induced by etoposide in HER2/neu-overexpressing cancer cell lines (A) but not in HER2/neu low-expressing cancer cell lines (B). HER2/neu-overexpressing cancer cell lines or HER2/neu low-expressing cancer cell lines were mock infected or infected with either rAd-V or rAd-T/t at appropriate MOI, such that >90% of the cells were infected. For each cell line, the infection efficiency by rAd-V and rAd-T/t was similar. The infected cells were then subjected to soft-agarose colony formation assay as described in Materials and Methods. The colony-forming ability of rAd-V-infected cells was set at 100% and the ability of rAd-T/t-infected cells was expressed relative to this value. Columns, average of three independent experiments; bars, SD.

Figure 6. Soft-agarose colony formation assay of rAd-V- or rAd-T/t-infected HER2/neu-overexpressing cancer cell lines (A) and HER2/neu low-expressing cancer cell lines (B). HER2/neu-overexpressing cancer cell lines or HER2/neu low-expressing cancer cell lines were infected with either rAd-V or rAd-T/t at appropriate MOI, such that >90% of the cells were infected. For each cell line, the infection efficiency by rAd-V and rAd-T/t was similar. The infected cells were then subjected to soft-agarose colony formation assay as described in Materials and Methods. The colony-forming ability of rAd-V-infected cells was set at 100% and the ability of rAd-T/t-infected cells was expressed relative to this value. Columns, average of three independent experiments; bars, SD.

Figure 7. T/t-common specifically enhanced apoptosis induced by etoposide in HER2/neu-overexpressing cancer cell lines (A) but not in HER2/neu low-expressing cancer cell lines (B). HER2/neu-overexpressing cancer cell lines or HER2/neu low-expressing cancer cell lines were mock infected or infected with either rAd-V or rAd-T/t at appropriate MOI, such that >90% of the cells were infected. For each cell line, the infection efficiency by rAd-V and rAd-T/t was similar. Forty-eight hours later, cells were either treated with appropriate concentration of etoposide (10 μmol/L for BT-474; 15 μmol/L for SK-OV-3 and SK-OV-3.ip1; 5 μmol/L for SK-BR-3, NPC-TW04, HeLa, and HEp-2) or left untreated. After incubation in media containing 10% FBS for 48 more hours, cells were harvested and subjected to sub-G1 DNA content analysis. Columns, average of two independent experiments; bars, SD.
which T/t-common induces apoptosis in HER2/neu-overexpressing cancer cells. T/t-common expression leads to repression of HER2/neu, resulting in decreased activity of MEK1/2 and ERK1/2. This in turn results in down-regulation of Bcl-2 and Bcl-X₇, leading to apoptosis of the HER2/neu-overexpressing cancer cells. That down-regulation of Bcl-2 may play a role in T/t-common–induced apoptosis was also supported by the observation that introduction of Bcl-2 into T/t-common–transfected HER2/neu-overexpressing cancer cells could block the ability of T/t-common to kill these cells (Fig. 3B).

A number of studies have suggested that cancer cells are often physiologically dependent on the continued activity of specific activated or overexpressed oncogenes for maintenance of their malignant phenotype (47). These studies have led to a new concept with respect to cancer treatment; i.e., cancer cell might be more susceptible to therapies that target the oncogene critical for the development of the specific cancer. In this respect, it has been shown that gefitinib (Iressa), an EGFR tyrosine kinase inhibitor, is more effective in treatment of lung cancer patients carrying mutant EGFR with higher receptor activity in their tumors (48). It has been suggested that HER2/neu-overexpressing cancer cells are dependent on HER2/neu for survival and thus are sensitive to treatments that inhibit HER2/neu (34–36). In contrast, cancer cells that express low levels of HER2/neu have presumably undergone neoplastic transformation by distinct molecular mechanisms and thus are resistant to HER2/neu inhibitory agents. Because T/t-common can specifically inhibit HER2/neu, it is reasonable to assume that T/t-common may specifically suppress the tumorigenicity of HER2/neu-overexpressing cancer cells. Indeed, in this study, we showed that T/t-common not only can specifically induce apoptosis of HER2/neu-overexpressing cancer cells (Fig. 4) but also can specifically suppress the in vitro transforming ability of these cancer cells (Fig. 6).

T/t-common could efficiently induce apoptosis of HER2/neu-overexpressing cancer cells in low-serum medium but had much lower ability to do so in high-serum medium (Figs. 1 and 4; data not shown). The reason for this could be that under high-serum conditions, T/t-common cannot efficiently block the survival signals transduced by other ligand-activated HER receptors. The HER family members, including EGFR, HER3, and HER4, are activated on ligand-induced receptor homo- and heterodimerization (1, 2). The HER2/neu-overexpressing cancer cell lines we tested, in addition to overexpressing HER2/neu, also express significant amount of other HERs that bind specific ligands. For instance, SK-OV-3 cells also express fairly high level of EGFR; SK-BR-3 and AU565 cells express significant levels of EGFR and HER3; and MDA-MB-453 cells produce significant amounts of HER3 and HER4 (37, 38). These ligand-specific HERs may bind their cognate ligands presented in the serum and transduce survival signals. Thus, even when HER2/neu is blocked by T/t-common, the signals transduced by other ligand-activated HERs are enough to maintain cell survival. A significant fraction of T/t-common–transduced HER2/neu-overexpressing cancer cells die, nevertheless, under high-serum condition (Fig. 1B; data not shown). This is because HER2/neu is a preferred heterodimerization partner of other HERs (4) and inhibition of HER2/neu would block survival signals at a certain extent even at high-serum condition. In contrast, under low-serum condition, these HER2/neu-overexpressing cancer cells are primarily dependent on signals transduced by overexpressed HER2/neu, which does not bind any of the known ligands with high affinity. Thus, T/t-common can efficiently induce apoptosis of these HER2/neu-overexpressing cancer cells in low-serum medium.

We showed that rAd-T/t infection could specifically and significantly inhibit SK-OV-3, SK-OV-3p1, AU565, MDA-MB-453, and BT-474 colony formation in soft agarose containing high-serum medium. These results indicate that T/t-common can suppress the transforming ability of the HER2/neu-overexpressing cancer cells even under high-serum condition. The colony-forming assay in soft agarose measures anchorage-independent cell proliferation and survival. Apparently, T/t-common is very effective in inhibiting the proliferation and survival of HER2/neu-overexpressing cancer cells under anchorage-free condition. rAd-T/t infection most likely caused apoptotic death of these cancer cells in soft agarose because colony formation by these cancer cells was still inhibited long after T/t-common expression ceased (data not shown). We have shown that under high-serum condition, T/t-common could not efficiently induce apoptosis of HER2/neu-overexpressing cancer cells growing on tissue culture plates. Why could T/t-common effectively do so in soft agarose containing high-serum medium? It is possible that under anchorage-free condition, these HER2/neu-overexpressing cancer cells need more survival and proliferation signals transduced by HER2/neu and thus become more dependent on HER2/neu for survival. Hence, under stressed condition, such as growth in soft agarose, T/t-common would be more effective in activating apoptosis in HER2/neu-overexpressing cancer cells. In this regard, it is worth noting that Herceptin, a humanized monoclonal antibody against HER2/neu, can effectively inhibit colony formation by HER2/neu-overexpressing BT-474 breast cancer cells in soft agarose but cannot induce apoptosis of these cells on tissue culture plates (49). That T/t-common is more effective in activating apoptosis of HER2/neu-overexpressing cancer cells under stressed condition was also supported by the observation that T/t-common efficiently induced apoptosis of HER2/neu-overexpressing cancer cell lines in high-serum medium containing chemotherapeutic drug etoposide (Fig. 7A).

Previous studies have shown that HER2/neu-overexpressing cancer cells are resistant to chemotherapeutic drug etoposide (14, 43) and treatments that inhibit HER2/neu can sensitize these cancer cells to etoposide (44, 45). Here we show that T/t-common, an inhibitor of HER2/neu, could also enhance etoposide-induced apoptosis in HER2/neu-overexpressing cancer cell lines but not in HER2/neu low-expressing cancer cell lines (Fig. 7). In addition, we also found that T/t-common could sensitize HER2/neu-overexpressing cancer cells, but not HER2/neu low-expressing cancer cells, to other chemotherapeutic agents, such as cisplatin, doxorubicin, and paclitaxel (data not shown). These data suggest that T/t-common gene therapy in combination with chemotherapy may provide enhanced therapeutic efficacy and specificity in the treatment of HER2/neu-overexpressing tumors.

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Induction of Apoptosis by SV40 T-Common

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SV40 T/t-Common Polypeptide Specifically Induces Apoptosis in Human Cancer Cells that Overexpress HER2/neu

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