24 hr, the detachment of round shaped cells and autophagic markers were induced at 48 hr and more increased at 72 hr after infection. At 72 hr after infection, autophagic vesicles were also confirmed in the OBP-301-infected H1299 and A549 cells under electron microscopy. Furthermore, we found the significant correlations between cytopathic effect of OBP-301 and autophagy-related markers, such as LC3-II/LC3-I ratio and expressions of Atg5 and p62 (Fig. 1*c*). These results suggest that there is a relationship between the cytopathic activity of OBP-301 and induction of autophagy.

Autophagy is well known to show both cytoprotective and cytopathic effects in human cancer cells. Autophagy has recently been shown to suppress tumorigenesis through p62 downregulation.[24] Furthermore, the accumulation of p62 proteins has been shown to be a critical factor for the survival of human cancer cells.[25] We, therefore, determined if OBP-301-mediated autophagy, which leads to downregulation of p62, is associated with cell survival or cell death. For this purpose, we analyzed the effect of siRNA-mediated downregulation of p62, which mimics OBP-301-mediated p62 downregulation, on the viability of noninfected H1299, A549 and T.Tn cells (Supporting Information Fig. 5). Using Western blotting analysis, we first confirmed complete suppression of p62 protein expression by transfection of 10 nM *p62* siRNA in all cell lines (Supporting Information Fig. 5a). Treatment with p62 siRNA significantly suppressed cell viability in all cell lines in a dose-dependent manner (Supporting Information Fig. 5b), suggesting that OBP-301-mediated downregulation of p62 induces cell death. In contrast to OBP-301-induced autophagic cell death, no apoptotic cell death, assessed by western blotting of caspase-3 cleavage, was observed in OBP-301-infected H1299 and A549 cells (Supporting Information Fig. 6). These results suggest that the cytopathic effect of OBP-301 is associated with autophagy-related cell death.

OBP-301 infection modulates miRNA expression in human cancer cells

To next investigate if OBP-301 induces autophagic cell death through modulation of miRNA expression in human cancer cells, OBP-301-sensitive H1299 cells were infected with OBP-301, and miRNA expression levels in the OBP-301-infected and mock-infected cells were analyzed using a miRNA microarray. Because wild-type Ad5 is the virus from which OBP-301 was generated, an Ad5-infected H1299 cell extract was also analyzed to clarify the candidate miRNAs modulated by infection with OBP-301 and/or Ad5. Fifteen miRNAs showed differences in expression that were higher than 50% in the OBP-301-treated and/or Ad5-treated cells compared to mock-treated cells (Supporting Information Fig. 7a). Of these 15 miRNAs, four miRNAs were downregulated and 11 miRNAs were upregulated. To further validate OBP-301-mediated modulation of miRNA expression, we further analyzed four miRNAs; two downregulated miRNAs (*miR-33a* and *miR-183*) and two upregulated miRNAs (*miR-483-3p* and *miR-7*), using same three RNA samples used for miRNA microarray by quantitative real-time RT-PCR (qRT-PCR) (Supporting Information Fig. 7b). Of these four miRNAs, the expression of *miR-7* was upregulated 2.94-fold and 1.91-fold in the OBP-301-treated and Ad5-treated cells, respectively, compared to mock-treated cells. This result for *miR-7* was consistent with the microarray data, whereas other three miRNAs showed different expression levels between microarray and qRT-PCR. Therefore, for further analysis, we focused on the role of *miR-7* in OBP-301-mediated oncolytic cell death.

miR-7 upregulation is associated with the cytopathic activity of OBP-301

To further confirm OBP-301-mediated *miR-7* upregulation, OBP-301-sensitive (H1299 and A549) and OBP-301-resistant (T.Tn and NHLF) cells were infected with OBP-301 at various MOIs, and the expression level of *miR-7* was examined using qRT-PCR. *miR-7* expression was dose-dependently upregulated in the OBP-301-infected H1299 and A549 cells, whereas T.Tn and NHLF cells showed no change in *miR-7* expression after OBP-301 infection (Fig. 2*a*). Time-dependent upregulation of *miR-7* expression was also observed in H1299 and A549 cells infected with OBP-301 at 5 and 50 MOIs, respectively (Supporting Information Fig. 8a). Furthermore, the level of *miR-7* upregulation after OBP-301 infection significantly correlated with the cytopathic activity of OBP-301 ($r = 0.954$, $p = 5.78\text{E-}13$) (Supporting Information Fig. 8b). Similar to OBP-301, Ad5 infection also dose-dependently upregulated *miR-7* expression, and this upregulation significantly correlated with the cytopathic activity of Ad5 ($r = 0.933$, $p = 8.94\text{E-}6$) (Supporting Information Fig. 9). These results suggest that *miR-7* upregulation is implicated in oncolytic adenovirus-mediated cell death.

E2F1 activation is involved in OBP-301-mediated miR-7 upregulation

Adenovirus infection has been shown to modulate many kinds of protein-coding genes through activation of the transcription factor, E2F1, induced by adenoviral E1A[26] and E4.[27,28] Furthermore, it has recently been shown that E2F1 regulates the expression of specific miRNAs in a transcription-dependent manner.[29] Therefore, we sought to assess the role of E2F1 in OBP-301-mediated *miR-7* upregulation. The Western blotting analysis revealed that OBP-301 infection at MOIs greater than five induced E2F1 protein expression in OBP-301-sensitive H1299 and A549 cells but not in OBP-301-resistant T.Tn cells (Fig. 2*b*). In contrast, NHLF cells showed slight increase in E2F1 expression after infection with high dose (more than 50 MOI) of OBP-301.The level of *miR-7* upregulation in these cells significantly correlated with the level of E2F1 expression ($r = 0.944$, $p = 4.48\text{E-}12$) (Supporting Information Fig. 8c). Furthermore, to investigate the E2F1-mediated *miR-7* upregulation, H1299 and A549 cells were infected with or without an E2F1-expressing replication-deficient adenoviral vector (Ad-E2F1) (100 MOI) for

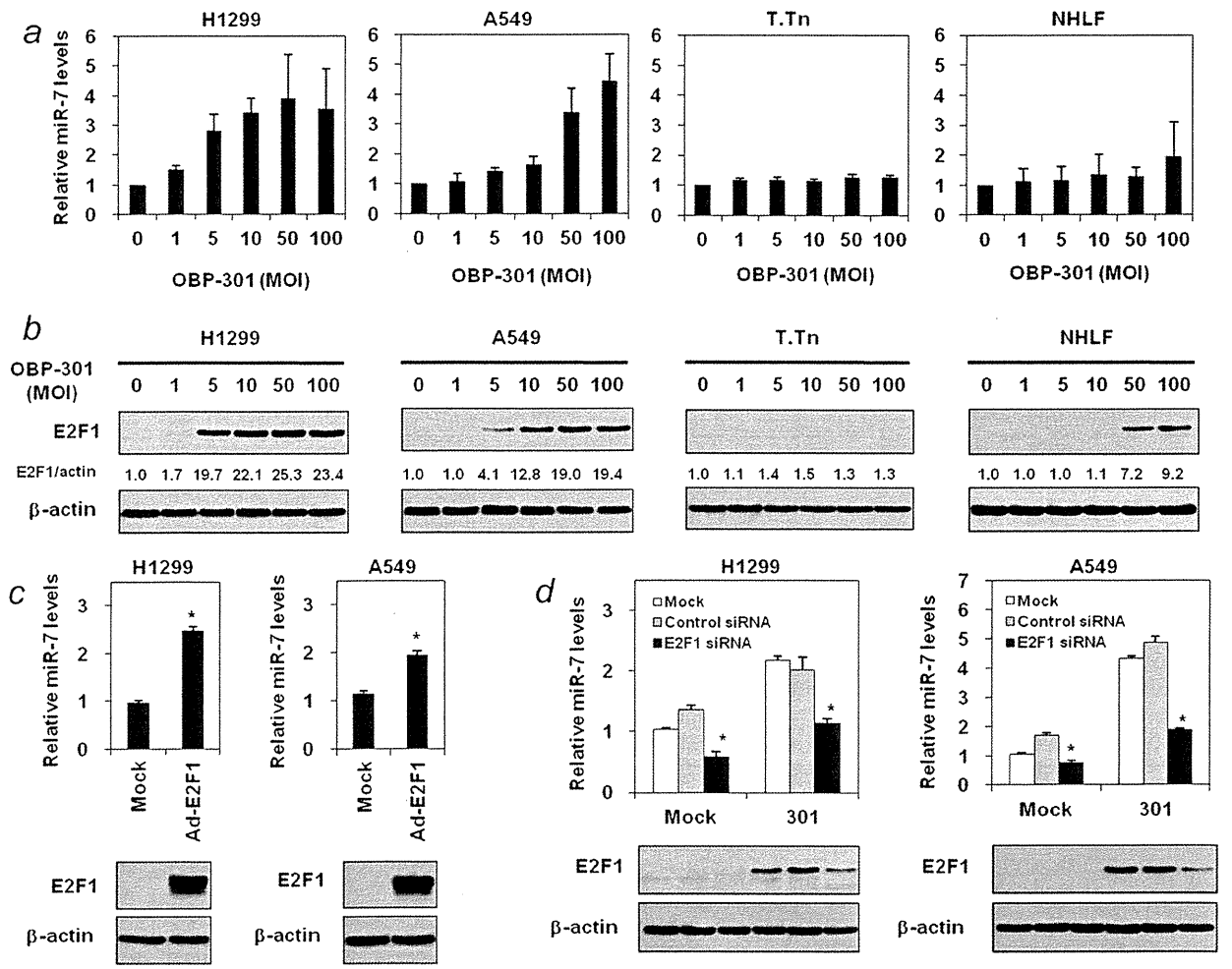

Figure 2. OBP-301-induced E2F1 expression upregulates *miR-7* expression in association with cytopathic activity in human cancer cells. (*a*) *miR-7* expression was assayed using qRT-PCR in H1299, A549, T.Tn and NHLF cells infected with OBP-301 at the indicated MOIs for 72 hr. The values of *miR-7* at 0 MOI and 0 hr were set at 1, and the relative levels of *miR-7* at the indicated MOIs and time points were plotted as fold induction. *miR-7* expression data are expressed as mean values ± SD (n = 3). (*b*) Western blot analysis of the expression of the E2F1 protein in H1299, A549, T.Tn and NHLF cells infected with OBP-301 at the indicated MOIs for 72 hr. E2F1 expression levels were calculated relative to actin expression in the same sample. E2F1/actin expression values at 0 MOI were set at 1.0. Relative levels of E2F1 expression were calculated as fold induction. (*c*) *miR-7* expression in H1299 and A549 cells infected with E2F1-expressing adenoviral vectors for 48 hr was assessed using qRT-PCR. The values of *miR-7* expression in the mock-infected cells were set at 1, and the relative levels of *miR-7* in the E2F1-overexpressing cells were plotted as fold induction. Expression of the E2F1 protein was confirmed by Western blot analysis using actin as a loading control. (*d*) *miR-7* and E2F1 expression in H1299 and A549 cells that were mock-pretreated or were pretreated with control siRNA or with E2F1 siRNA (10 nM) before mock-infection or to infection with OBP-301 at 5 and 50 MOIs, respectively. The values of *miR-7* expression in the mock-infected cells without pretreatment were set at 1, and relative levels of *miR-7* were plotted as fold induction. Expression of the E2F1 protein was confirmed by Western blot analysis. β-Actin was used as a loading control. For (*c*) and (*d*), *miR-7* expression data are expressed as mean values ± SD ($n = 3$). Statistical significance (*) was defined as $p < 0.05$.

48 hr. However, E1A-deleted control adenovirus was not used because other genes from E4 region might contribute to E2F1 activation.[27,28] Ectopic expression of E2F1 by infection with Ad-E2F1 significantly upregulated *miR-7* expression 2.48- and 1.96-fold in H1299 and A549 cells, respectively, compared to mock infection (Fig. 2*c*). Overexpression of the E2F1 protein by Ad-E2F1 infection was confirmed by Western blot analysis. Conversely, specific downregulation of E2F1 by pretransfection of E2F1 siRNA (10 nM) significantly suppressed the level of *miR-7* expression compared to mock or control siRNA treatment in both mock-infected and OBP-301-infected cells at 72 hr after OBP-301 infection following to siRNA treatment for 24 hr (Fig. 2*d*). Suppression of OBP-301-activation of E2F1 expression by pretreatment with E2F1

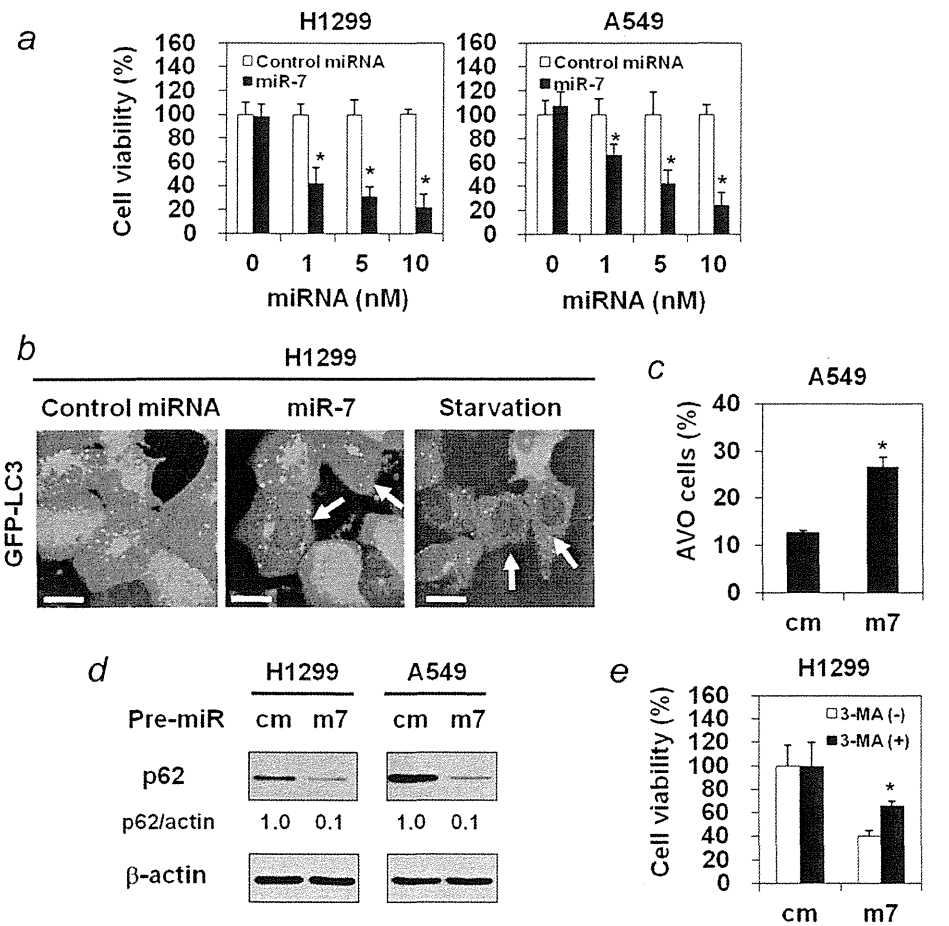

Figure 3. Induction of autophagy by *miR-7* overexpression. (*a*) The effect of transfection of the indicated concentrations of control miRNA (white bars) or *miR-7* (black bars) on the cell viability of H1299 and A549 cells was determined using XTT assay on day 6 after transfection. Cell viability of *miR-7*-transfected cells was calculated relative to that of control miRNA-transfected cells, which was set at 100%. (*b*) Immunofluorescence analysis of H1299 cells stably expressing the green fluorescent protein (GFP)-microtubule-associated protein 1 light chain 3 (GFP-LC3) fusion protein and transfected with 50 nM *miR-7* or control miRNA for 3 days. Serum-starved H1299-GFP-LC3 cells were used as a positive control. The white arrows indicate autophagic cells with punctate GFP expression in the cytoplasm. Scale bars: 50 μm. (*c*) Acidic vesicular organelles (AVO), present in A549 cells transfected with 5 nM *miR-7* (m7) or control miRNA (cm) for 3 days, were quantified by staining with acridine orange followed by flowcytometric analysis. The percentage of AVO cells, which are indicative of autophagy, are expressed as mean values ± SD ($n = 3$). (*d*) Expression of the p62 protein in H1299 and A549 cells transfected with 10 nM *miR-7* (m7) or control miRNA (cm) for 3 days was assayed by Western blotting. β-actin was assayed as a loading control. p62 expression levels were calculated relative to actin expression in the same sample. p62 expression levels were then calculated relative to the p62 expression levels of control miRNA-transfected cells, which were set at 1.0. (*e*) The viability of H1299 cells, transfected with 10 nM *miR-7* (m7) or with control miRNA (cm) following pretreatment with or without the autophagy inhibitor 3-methyladenine (3-MA), was assayed using the XTT assay. Cell viability data are expressed as mean values ± SD ($n = 5$). Statistical significance (*) was defined as $p < 0.05$.

siRNA was also confirmed by Western blot analysis. These results suggest that OBP-301-mediated *miR-7* upregulation depends mainly on activation of E2F1 expression.

miR-7 overexpression suppresses cell viability through induction of autophagy in human cancer cells

To determine if OBP-301-mediated *miR-7* overexpression is associated with cell death in human cancer cells, we introduced exogenous *miR-7*, or control miRNA, into OBP-301-sensitive H1299, A549 and T.Tn cells and investigated the effect of *miR-7* overexpression on cell viability (Fig. 3*a* and Supporting Information Fig. 10a). Ectopic expression of *miR-7* significantly suppressed cell viability in a dose-dependent manner compared to control miRNA in H1299 and A549 cells. However, T.Tn cells showed less sensitivity to *miR-7*-mediated suppression of cell viability compared to H1299 and A549 cells. In contrast, *miR-7*-overexpression did not induce apoptotic cell death (caspase-3 cleavage) in any of

these cells (Supporting Information Fig. 11). These results indicate that *miR-7* overexpression suppresses cell viability through induction of nonapoptotic cell death in human cancer cells.

The observation of *miR-7*-mediated nonapoptotic cell death prompted us to investigate if *miR-7* overexpression induces autophagic cell death, because we had previously shown that autophagy may be involved in OBP-301-mediated oncolysis of H1299 cells[30] and observed the significant correlation between cytopathic activity of OBP-301 and autophagy induction (Fig. 1). To investigate *miR-7*-mediated induction of autophagy in cells, we used H1299 cells that were stably transfected with a GFP-LC3 fusion plasmid (GFP-LC3). *miR-7* overexpression in H1299-GFP-LC3 cells induced the appearance of autophagic cells with a punctate pattern of GFP-LC3 expression in the cytoplasm, similar to that observed in serum-starved cells (Fig. 3*b*). To quantify autophagy induced by *miR-7*, A549 cells that were transfected with *miR-7* or control miRNA were stained with acridine orange. The percentage of cells with stained acidic vesicular organelles (AVOs), which are indicative of autophagy, was then measured using flow cytometry. Transfection of A549 cells with *miR-7* significantly increased the percentage of AVO-positive cells compared to control miRNA (Fig. 3*c*). These results indicate that *miR-7* overexpression induces autophagy.

Because OBP-301 infection both upregulates *miR-7* and downregulates p62 in association with autophagy, we determined if *miR-7* overexpression might induce downregulation of p62 protein expression. *miR-7* transfection suppressed p62 expression compared to control miRNA in H1299 and A549 cells as shown by Western blotting (Fig. 3*d*). These results indicate that *miR-7* overexpression induces autophagy, resulting in p62 downregulation, in human cancer cells. To further examine if *miR-7* overexpression suppresses cell viability through induction of autophagy, the effect of the autophagy inhibitor, 3-MA, on *miR-7*-mediated suppression of cell viability was determined. Treatment of H1299 cells with 3-MA significantly attenuated *miR-7*-mediated suppression of cell viability (Fig. 3*e*), suggesting that *miR-7* does indeed mediate autophagic cell death.

EGFR downregulation by miR-7 overexpression is implicated in the OBP-301-mediated cytopathic effect

Recent evidence had shown that *miR-7* functions as a tumor suppressor by suppressing the expression of the epidermal growth factor receptor (EGFR),[31,32] which is strongly associated with tumor progression and poor prognosis in human cancers.[33] Furthermore, a recent report has shown that EGFR downregulation by siRNA induces autophagic cell death in human cancer cells.[34] We, therefore, next sought to determine if OBP-301 suppresses EGFR expression through *miR-7* upregulation. As shown in Figure 4*a*, OBP-301 infection suppressed EGFR expression in a dose-dependent manner in OBP-301-sensitive H1299 and A549 cells but not in OBP-301-resistant T.Tn and NHLF cells. The level of EGFR suppression was significantly associated with the level of *miR-7* upregulation ($r = -0.872$, $p = 2.64E-8$) (Fig. 4*b*) and with the cytopathic activity of OBP-301 ($r = -0.826$, $p = 6.73E-7$) (Fig. 4*c*), suggesting the involvement of *miR-7*-mediated EGFR suppression in the cytopathic effect of OBP-301. The ectopic expression of *miR-7* suppressed EGFR expression compared to control miRNA in H1299 and A549 cells (Fig. 4*d*). Furthermore, ectopic expression of E2F1 by infection with an Ad-E2F1 also downregulated EGFR expression compared to mock infection in H1299 and A549 cells (Fig. 4*e*). In contrast, treatment with EGFR siRNA significantly suppressed cell viability compared to control siRNA in H1299 and A549 cells (Fig. 4*f*). However, *miR-7*-resistant T.Tn cells showed about fivefold higher expression level of EGFR compared to H1299 and A549 cells (Supporting Information Fig. 10b). Even when T.Tn cells were transfected with *miR-7* at 10 nM, high expression levels of EGFR and p62 were maintained. The combined results suggest that OBP-301 infection induces *miR-7* expression through E2F1 activation and that E2F1-mediated *miR-7* upregulation suppresses EGFR expression, resulting in the induction of autophagy-related cell death (Fig. 4*g*).

Discussion

Tumor-specific replication-competent oncolytic virotherapy is emerging as a promising anticancer therapy for the induction of tumor-specific oncolytic cell death.[4] Although the possible involvement of autophagy in oncolytic adenovirus-mediated cell death has recently been suggested,[5–9] the molecular mechanism by which autophagic cell death is induced remains to be elucidated. In this study, we demonstrated that infection with the oncolytic adenovirus, OBP-301, upregulated *miR-7* expression and that this upregulation was associated with its cytopathic activity in human cancer cells. Furthermore, OBP-301-mediated E2F1 activation was involved in *miR-7* upregulation, which subsequently induced autophagy through suppression of EGFR expression in human cancer cells. Adenovirus infection is well known to induce the viral protein-mediated E2F1 activation and subsequent upregulation of many E2F1-target genes.[35] Recently, E2F1 has been shown to induce autophagy through upregulation of autophagy-related genes in a transcription-dependent manner.[36] In contrast, EGF is known to suppress autophagy through EGFR activation.[37] Furthermore, it has been shown that EGFR downregulation by EGFR siRNA causes autophagic cell death in human cancer cells.[34] Thus, oncolytic adenoviruses may activate E2F1 expression, resulting in the upregulation of autophagy-related genes and the downregulation of autophagy-suppressing genes via miRNA modulation. Subsequently, autophagy-related programmed cell death is induced.

OBP-301 induced higher levels of autophagy, replication rate and cytopathic activity than Ad5 in H1299 and A549 cells (Fig. 1 and Supporting Information Figs. 2 and 3).

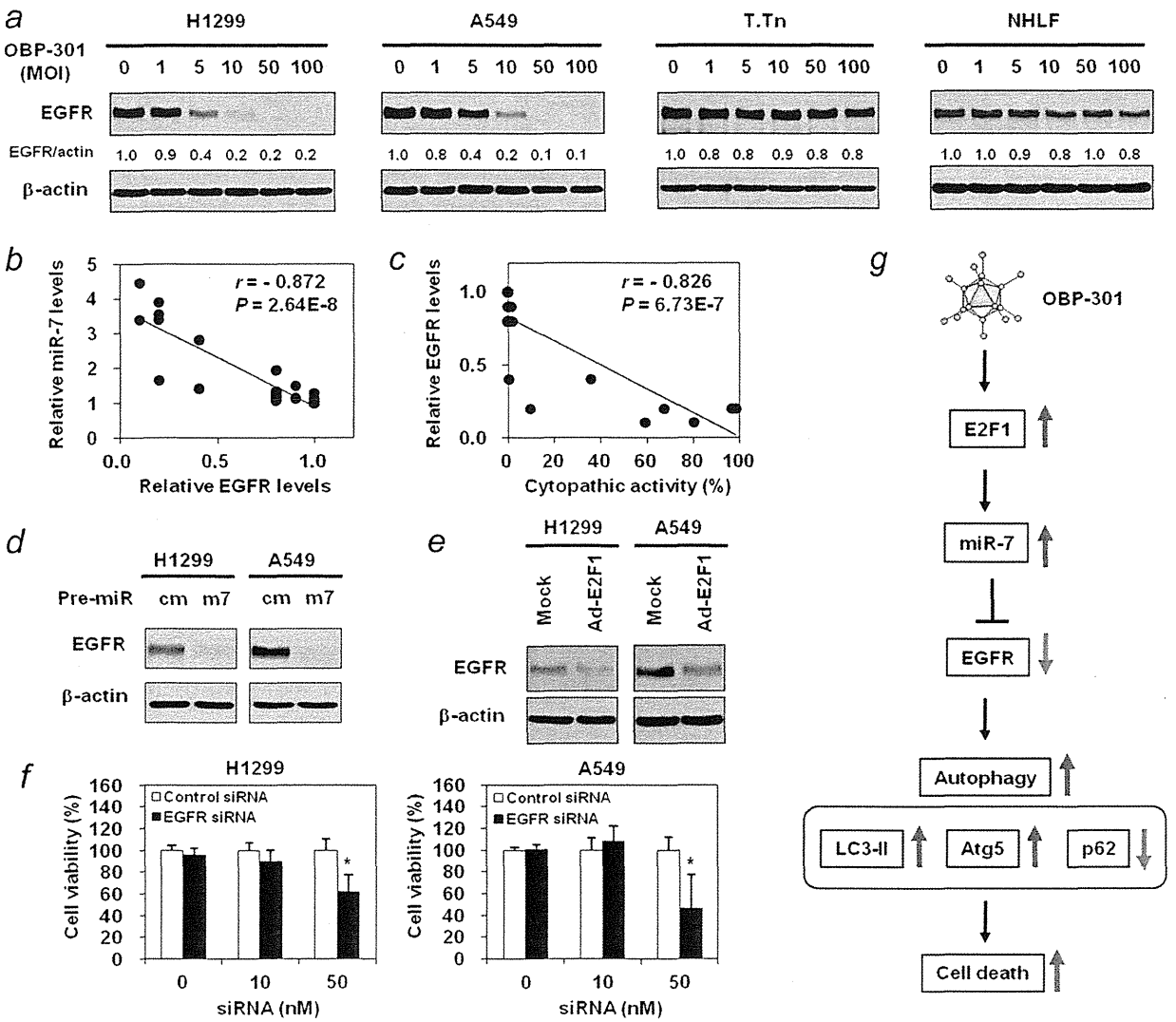

Figure 4. *miR-7*-mediated suppression of EGFR expression during OBP-301-mediated autophagic cell death. (*a*) Western blot analysis of EGFR protein expression in H1299, A549, T.Tn and NHLF cells infected with OBP-301 at the indicated MOIs for 72 h. EGFR expression levels were calculated relative to actin expression in the same sample. EGFR expression in OBP-301 infected cells was then calculated relative to that in mock-infected cells, whose value was set at 1. (*b*) There was a significant correlation between the expression level of EGFR and that of *miR-7*. (*c*) There was a significant correlation between the expression level of EGFR and the cytopathic activity of OBP-301. (*d*) Western blot analysis of the expression of the EGFR protein in H1299 and A549 cells transfected with 10 nM *miR-7* (m7) or with control miRNA (cm) for 72 hr. EGFR expression in the m7-transfected cell was calculated relative to that in the cm-transfected cells. β-actin was assayed as a loading control. (*e*) Western blot analysis of the expression of the EGFR protein in H1299 and A549 cells infected with E2F1-expressing adenoviral vectors for 48 hr. (*f*) Effect of EGFR siRNA transfection on the viability of H1299 and A549 cells. Cell viability was determined on day 6 after transfection using the XTT assay. Cell viability was calculated relative to that of mock-transfected cells, whose viability was set at 100%. Cell viability data are expressed as mean values ± SD ($n = 5$). Statistical significance (*) was defined as $p < 0.05$. (*g*) Outline of the E2F1-*miR-7*-EGFR axis during OBP-301-mediated autophagy.

Recent report has shown that adenovirus-mediated autophagy induction is associated with viral replication and oncolysis.[38] Autophagy inhibitor 3-MA has been suggested to inhibit the replication rate of Ad5 in A549 cells.[38] However, our collaborators have shown that pretreatment with 3-MA or Atg5 siRNA did not affect the replication and oncolysis of fiber-modified OBP-301 in human brain tumor cells.[22] Recently, we observed that OBP-301 infection upregulated *hTERT* mRNA expression and, subsequently, showed higher levels of replication rate and oncolysis than Ad5 in human sarcoma cells.[39] Therefore, the replication of OBP-301 may be less sensitive to autophagy inhibitor compared to Ad5 because of enhanced viral replication by *hTERT* promoter activation.

On the molecular mechanism of adenovirus-induced oncolysis, recent report has suggested that adenovirus-mediated autophagy induces caspase-8 activation in association with oncolysis in human leukemia cells and normal fibroblasts.[40] Recently, caspase-8 has been shown to be involved in not only apoptosis but also diverse cell fates including autophagy.[41] In this study, we observed that oncolytic adenovirus induces autophagic cell death, not apoptotic cell death, in human cancer cells (Fig. 1 and Supporting Information Fig. 6). These results suggest the functional role of caspase-8 in adenovirus-mediated autophagic cell death. Atg5-mediated autophagic cell death has recently been shown to be induced through interaction with Atg5 and Fas-associated protein with death domain (FADD),[42] which can also bind with caspase-8.[41] Thus, oncolytic adenovirus may contributes to autophagic cell death through activation of Atg5-FADD-caspase-8 network. Furthermore, although transfection with p62 siRNA suppressed p62 expression more strongly than OBP-301, the inhibitory effect of p62 siRNA was lower than OBP-301 in the cell viability of H1299, A549 and T.Tn cells (Fig. 1 and Supporting Information Fig. 5). These results suggest that OBP-301-mediated p62 downregulation not only suppresses oncogenic p62 function but also contributes to autophagy-related cell death.

We demonstrated that OBP-301-mediated activation of E2F1 expression upregulated *miR-7* expression in human cancer cells. E2F1 has recently been shown to regulate both oncogenic and tumor-suppressive miRNAs. The cluster of oncogenic miRNAs in the *miR-19-72* polycistron has been shown to be upregulated by E2F1.[43] In contrast, E2F1-inducible *miR-449a/b* has been shown to suppress cell proliferation and to induce apoptosis in human cancer cells.[44] Furthermore, Brosh *et al.* have suggested that 15 p53-repressed miRNAs, including the *miR-19-72* cluster and *miR-7*, are possibly regulated by E2F1,[45] which is consistent with our results that show E2F1-mediated *miR-7* upregulation. We previously reported that p53-inducible *miR-34a* suppresses E2F1 protein expression, resulting in downregulation of the E2F signaling pathway in human cancer cells.[13] These reports suggest possible cross-talk between E2F1, p53 and miRNAs. As adenovirus infection is well known to induce E2F1 expression,[35] but to suppress p53 expression,[46] the E2F1-inducible miRNA network may mainly assist the induction of autophagic cell death by oncolytic adenoviruses.

OBP-301-resistant T.Tn cells showed no induction of the E2F1-*miR-7*-EGFR axis, resulting in a lack of OBP-301-mediated autophagic cell death. Adenovirus infection is known to modulate E2F1 expression via two main viral factors, E1A and E4. E1A interacts with the phosphorylated retinoblastoma protein, resulting in the release of free E2F1.[26] In contrast, the adenoviral E4 19 kDa protein has been shown to enhance E2F1 protein levels through inhibition of proteasome-mediated E2F1 degradation.[27,28] Although the molecular basis for the lack of OBP-301-mediated E2F1 activation in T.Tn cells remains unclear, the cytopathic effect of an oncolytic adenovirus may mainly depend on E2F1 activation, leading to induction of autophagic cell death via modulation of E2F1-downstream target genes including miRNAs. On the role of another E2F family members during adenovirus infection, recent reports have suggested that adenovirus infection increases the E2F2 expression at the transcriptional level,[47] whereas the E2F4 expression is decreased.[48] Because it has been known that E2F2 is a transactivator as same as E2F1, but E2F4 functions as a transcriptional repressor, these E2F family members may function to induce the E2F-target gene network. Thus, further studies to address the role of E2F family members in OBP-301-mediated oncolytic cell death are warranted.

It has been recently shown that *miR-7* functions as a tumor suppressive miRNA by suppressing the expression of various EGFR signaling-related genes including that of *EGFR*, *insulin receptor substrate-2*, *Raf1* and *p21-activated kinase 1* in human cancer cells.[31,32,49] Consistent with these results, we observed that ectopic expression of *miR-7* suppressed cell proliferation and subsequently induced autophagic cell death through suppression of EGFR expression in human cancer cells. Regarding *miR-7*-mediated cell death, Webster *et al.* have suggested that nonapoptotic cell death is induced by *miR-7* transfection in human lung cancer A549 cells.[31] In contrast, Kefas *et al.* have shown that *miR-7* overexpression induces apoptotic cell death in human glioma cell lines.[32] These contradictory results suggest that *miR-7*-mediation of autophagic cell death may depend on the type of cancer cell in which it is expressed.

Overexpression or amplification of several types of EGFR gene isoforms is frequently observed in human cancers.[33] Recently, EGFR-targeting anticancer therapies, such as monoclonal antibodies and small molecule tyrosine kinase inhibitors, have been used to improve the clinical outcome of cancer patients. However, resistance to EGFR-targeting therapies is an issue that needs to be resolved. Furthermore, it has been recently reported that the EGFR regulates glucose transport that is required for the survival of cancer cells in an EGFR-kinase-independent manner.[34] This result suggests that not only inhibition of EGFR-kinase activity but also downregulation of the EGFR itself will be required for complete eradication of cancer cells. Recent report has further suggested that combination therapy of EGFR kinase inhibitor erlotinib with autophagy inducer rapamycin synergistically decreased the cell viability through increased autophagy in H1299 and A549 cells.[50] Our collaborators have also demonstrated that combination therapy of rapamycin with OBP-301 showed synergistic antitumor effect through activation of autophagy machinery in human brain tumor cells.[22] Taking the oncolytic adenovirus-mediated EGFR suppression and autophagy via *miR-7* induction into consideration, combination therapy of oncolytic adenoviruses with rapamycin may provide novel anticancer strategies that potentially have antitumor effects against cancer cells that are resistant to EGFR-targeting therapies.

In conclusion, we provide evidence, for the first time, that an oncolytic adenovirus induces autophagic cell death in human cancer cells through induction of *miR-7* upregulation via enhancement of E2F1 expression and through suppression of oncogenic EGFR expression. An understanding of oncolytic adenovirus-mediated modulation of the cellular miRNA network would provide novel insights into the antitumor mechanism of oncolytic virotherapy.

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BASIC STUDY

Wilms Tumor Gene 1 (WT1) Peptide–based Cancer Vaccine Combined With Gemcitabine for Patients With Advanced Pancreatic Cancer

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Summary: Wilms tumor gene 1 (WT1) protein is an attractive target for cancer immunotherapy. We aimed to investigate the feasibility of a combination therapy consisting of gemcitabine and WT1 peptide–based vaccine for patients with advanced pancreatic cancer and to make initial assessments of its clinical efficacy and immunologic response. Thirty-two HLA-A*24:02 $^{+}$ patients with advanced pancreatic cancer were enrolled. Patients received HLA-A*24:02-restricted, modified 9-mer WT1 peptide (3 mg/body) emulsified with Montanide ISA51 adjuvant (WT1 vaccine) intradermally biweekly and gemcitabine (1000 mg/m^2) on days 1, 8, and 15 of a 28-day cycle. This combination therapy was well tolerated. The frequencies of grade 3–4 adverse events for this combination therapy were similar to those for gemcitabine alone. Objective response rate was 20.0% (6/30 evaluable patients). Median survival time and 1-year survival rate were 8.1 months and 29%, respectively. The association between longer survival and positive delayed-type hypersensitivity to WT1 peptide was statistically significant, and longer survivors featured a higher frequency of memory-phenotype WT1-specific cytotoxic T lymphocytes both before and after treatment. WT1 vaccine in combination with gemcitabine was well tolerated for patients with advanced pancreatic cancer. Delayed-type hypersensitivity-positivity to WT1 peptide and a higher frequency of memory-phenotype WT1-specific cytotoxic T lymphocytes could be useful prognostic markers for survival in the combination therapy with gemcitabine and WT1 vaccine. Further clinical investigation is warranted to determine the effectiveness of this combination therapy.

Key Words: Wilms tumor gene (WT1), WT1 peptide vaccine, cancer immunotherapy, pancreatic cancer, gemcitabine

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Pancreatic cancer remains a malignancy with high mortality.[1] Gemcitabine has been the standard first-line treatment for patients with advanced pancreatic cancer, but featured a median overall survival time (MST) of about 6 months and a 1-year overall survival (OS) rate of $\leq$20%.[2] Although many trials of gemcitabine-based combination therapies with cytotoxic or biological agents have been attempted, these therapies, with the exception of erlotinib,[3] have not achieved any survival results superior to those attained with gemcitabine alone.[1] Prognosis of patients with pancreatic cancer thus remains extremely poor, so that novel treatments are urgently needed to improve survival.

Among promising therapeutic strategies, active cancer immunotherapies, such as peptide-based cancer vaccines against tumor-associated antigens (TAAs), which elicit TAA-specific cytotoxic T lymphocytes (CTLs) that eventually eradicate cancer cells, have been and are being developed.[4] However, because their clinical efficacy has been limited,[5,6] several approaches have been tried to improve their efficacy. One approach is the use of combination therapies with certain chemotherapeutic agents, including gemcitabine, which can stimulate the immune system.[7–9] An additional benefit is that chemotherapy makes the tumor cells susceptible to CTL response,[10,11] whereas cancer immunotherapy can sensitize the tumor cells to subsequent chemotherapeutic agents. For this reasons, cancer vaccine in combination with certain chemotherapeutic agents can be expected to exert synergistic effects.

The Wilms tumor gene (*WT1*) is highly expressed in various kinds of malignancies and has been found to perform oncogenic rather than tumor-suppressor functions in tumorigenesis.[12,13] Moreover, both cellular and humoral immune responses against the WT1 protein are naturally elicited in cancer patients, indicating that the *WT1* gene product is actually immunogenic.[14–18] In view of these

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findings, we and others have been performing clinical studies of the efficacy of WT1 peptide–based immunotherapies for patients, including children, with various kinds of malignancies.[13,19–26]

This report describes a phase 1 clinical study of a WT1 peptide–based cancer vaccine combined with gemcitabine for patients with advanced pancreatic cancer. The main objective of this study was to investigate the feasibility of this combination therapy and to make initial assessments of its clinical efficacy and the immunologic response to WT1 peptide.

MATERIALS AND METHODS

Patient Characteristics

Patients with pathologically or cytologically confirmed, measurable, locally advanced, or metastatic pancreatic adenocarcinoma or with recurrent disease were recruited for this noncomparative, open-label, phase 1 study at 2 centers: Osaka University Hospital and Jikei University Kashiwa Hospital, in Japan. Another major eligibility criterion was HLA-A*24:02 positivity. We chose this phenotype because about 60% of Japanese population had this phonotype. Other eligibility criteria included age of 20 years and older, 75 years and younger, Karnofsky performance status 60%–100%, no previous history of treatment for locally advanced or metastatic disease, a minimum 6-month interval from completion of any previous treatment for recurrent disease, a life expectancy of ≥ 3 months, and adequate organ functions. This study was approved by the ethical review boards of the 2 centers and performed in accordance with the Helsinki Declaration. All patients provided written informed consent.

WT Peptide–based Cancer Vaccine (WT1 Vaccine)

A HLA-A*24:02-restricted, modified 9-mer WT1 peptide (mp235; CYTWNQMNL; Peptide Institute Inc., Osaka, Japan) was generated according to the Good Manufacturing Practice Guidelines. In our previous report about the first clinical use of WT1 peptide,[19] the dose-escalation of WT1 peptide from 0.3 to 3.0 mg was designed to decide the recommended dose in combination with the incomplete Freund's adjuvant (Montanide ISA51; Seppic, Paris, France), and 3 mg of WT1 peptide in combination with Montanide ISA51 was decided to be well tolerated. In our present study, we chose WT1 vaccine composed of 3 mg of WT1 peptide and Montanide ISA51 adjuvant. WT1 vaccine was prepared, according to our previous report.[19] WT1 peptide of 3 mg was dissolved in a small volume of dimethyl sulfoxide (DMSO; Sigma, St Louis, MO). The solution was then diluted to 400 μL with 5% glucose and finally emulsified with an equal weight of Montanide ISA51 adjuvant.

Treatment

Gemcitabine was intravenously administered at a dose of 1000 mg/m^2 on days 1, 8, and 15 of a 28-day cycle. WT1 vaccine was intradermally administered at 6 different sites (bilateral upper arms, lower abdomen, and femoral regions) on days 1 and 15 of a 28-day cycle. The initial treatment protocol was planned as 2 courses. Patients without early progressive disease upon the completion of protocol treatment could receive additional treatment until the occurrence of disease progression, unacceptable adverse events, or withdrawal of consent.

Study Assessment

Toxicity was graded using the National Cancer Institute's Common Toxicity Criteria of Adverse Events (CTCAE version 3.0). Dose-limiting toxicity (DLT) was defined as the following adverse events, during the first 2 courses, which were possibly, probably, or definitely related to treatment: grade 4 hematological toxicity lasting >7 days, grade 3 or worse neutropenia accompanied by high fever (≥38°C) or infection (febril neutropenia), and any nonhematological toxicity of grade 3 or worse in other organ systems, including vaccine-injection sites. Biliary tract infection secondary to biliary obstruction was not considered to be a DLT unless it occurred in conjunction with grade ≥ 3 neutropenia. Computed tomography was performed every 4 weeks during the protocol treatment and

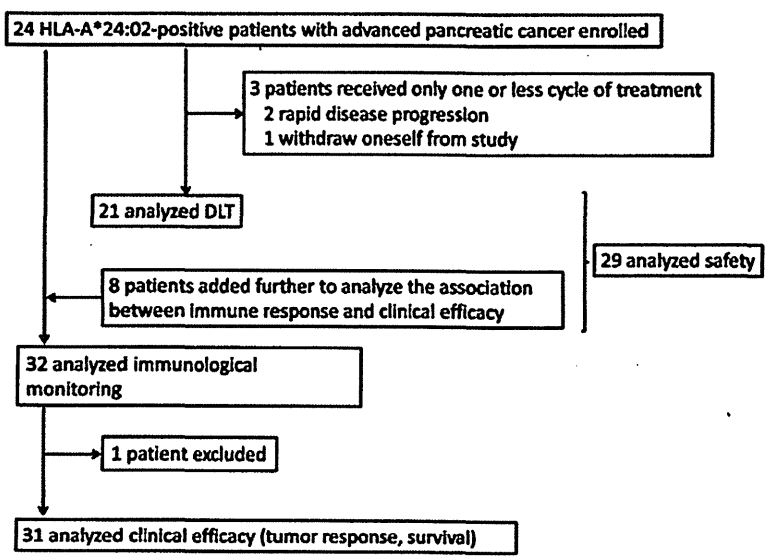

FIGURE 1. Study profile.

every 6–8 weeks during the additional treatment until disease progression, and tumor response was assessed by the investigators according to the Response Evaluation Criteria in Solid Tumors criteria. Stable disease (SD) was defined as a disease that was stable for >8 weeks after the beginning of treatment. The concentration of the tumor marker carbohydrate antigen 19-9 (CA19-9) was measured at baseline and each course.

WT1-specific Immunologic Assessment

As WT1-specific immunologic assessment, delayed-type hypersensitivity (DTH) to WT1 peptide and the WT1 peptide/HLA-A*24:02 tetramer assay was examined. DTH was examined on day 1 of each course during the protocol treatment and optionally at suitable time during the additional treatment. All DTH tests were performed and measured by the investigators. Briefly, 30 μg of WT1 peptide in saline and saline alone were intradermally injected in the forearm, and the maximum diameter of erythema and other skin reaction, including induration, were measured after 48 hours. DTH-positivity was defined as erythema >2 mm in diameter, which size was the minimum size measurable with a ruler at the clinical practice.

Peripheral blood (PB) mononuclear cells for WT1 peptide/HLA-A*24:02 tetramer assay were collected on day 1 of each course during the protocol treatment and appropriately during the additional treatment, and cryopreserved until use. The following tetramer and monoclonal antibodies were used: PE-conjugated $WT1_{235}$ tetramer [HLA-A*24:02-restricted natural 9-mer WT1 peptide (CMT WNQMNL)] (MBL, Nagoya, Japan), anti-CD4-FITC, anti-CD16-FITC, anti-CD45RA-APC (BioLegend, San Diego, CA), anti-CD19-FITC, anti-CCR7-PE-Cy7 (BD Pharmingen, San Diego, CA), anti-CD3-PerCP, anti-CD8-APC-Cy7, anti-CD14-FITC (BD Biosciences, San Jose, CA), and anti-CD56-FITC (eBioscience, San Diego, CA). Lineage antigen (CD4, CD14, CD16, CD19, and CD56)-negative, $CD3^-$, $CD8^-$, and $WT1_{235}$ tetramer$^+$ lymphocytes were defined as WT1 tetramer$^+$ $CD3^+$ $CD8^+$ T lymphocytes (WT1-CTLs). Data acquisition was performed on a FACS Aria instrument (BD Biosciences), and data analysis was performed with FACS Diva software (BD Biosciences).

Statistical Analysis

The safety profile constituted the primary end point. A treatment schedule was considered to be acceptable if the probability of developing DLT was estimated to be <20%. If the estimated probability of DLT occurrence was 10%, the upper limit of the 90% (1-sided) confidence interval (CI) of DLT probability was <20%, based on the projected sample size of 20 patients. For a more accurate determination of the associations with clinical efficacy and immunologic parameters, in total 32 patients were enrolled (8 patients were further enrolled after the completion of safety assessment with the initial 24 patients as shown in Figure 1). The secondary end points included objective response, CA19-9 response, defined as a decrease in CA19-9 concentration of at least 50% in the patients with >100 U/mL of CA19-9 at baseline, progression-free survival defined as time from date of beginning of the treatment to date of disease progression as confirmed by the investigators or death without progression, OS, immunologic responses to WT1 peptide, and correlations between clinical benefit response (CBR)[2] and quality of life (QOL) assessed using by the Functional Assessment of Cancer Therapy-General (FACT-G) measurement system.[39] The nonparametric, Wilcoxon signed-rank test or Mann-Whitney U test was used to calculate P values for change in immune cells because the data were skewed. We judged P values of <0.01 to be significant. χ^2 test was used to calculate P values for associations between DTH and clinical efficacy. The statistical analyses were performed with SAS for Windows version 9.2 (SAS Institute Inc., Cary, NC). Correlations between CBR and the physical and functional scores based on replies to the FACT-G QOL questionnaire were analyzed with a linear mixed-effects model, for which SAS for Windows release 9.1 (SAS Institute Inc.) was used.

RESULTS

Patient Characteristics

A total of 63 patients with advanced pancreatic cancer, whose median age was 63.0 years old, were screened and checked a phenotype in HLA-A locus. Twenty-two patients failed to enroll in this trial because of lack of HLA-A*24:02 phenotype. A total of 32 HLA-A*24:02$^+$ patients with advanced pancreatic cancer were finally enrolled in this trial between 2008 and 2010. Of 32 patients, 28 had inoperable advanced pancreatic cancer (6 locally advanced and 22 metastatic diseases), and the remaining 4 had recurrent

TABLE 1. Patients Characteristics at Baseline

Characteristics	N (%)
Age (y)	
Median	60.0
Range	41–75
Sex	
Male	17 (53.1)
Female	15 (46.9)
Karnofsky performance status (%)	
~70	7 (21.9)
80	10 (31.3)
90	12 (37.5)
100	3 (9.4)
Disease extent	
Inoperable advanced disease	28 (87.5)
Locally advanced	6 (18.8)
Metastatic	22 (68.8)
Recurrent disease	4 (12.5)
Local relapse	1 (3.1)
Distant metastasis	3 (9.4)
Primary tumor site	
Head	15 (46.9)
Body/tail	17 (53.1)
Metastatic sites	
Liver	17 (53.1)
Distant lymph node	16 (50.0)
Lung	7 (21.9)
Peritoneum	6 (18.8)
Others*	4 (12.5)
CA19-9 concentration at baseline (U/mL)	
Median	248
Range (U/mL)	<5–75,050
<5	3 (9.4)†
6–99	10 (31.3)
100–999	7 (21.9)
1000–9999	5 (15.6)
>10,000	7 (21.9)

*Other metastatic sites included bone, ovary, or adrenal gland.
†All patients had the Lewis blood group-negative phenotype.
CA19-9 indicates carbohydrate antigen 19-9.

disease. Table 1 summarizes the patient baseline characteristics. Three patients did not complete the first 2 courses of treatment: 2 patients showed rapid disease progression, and 1 refused to continue the treatment. It was determined by the supervising Data Safety and Monitoring Board that the elimination of these cases was unlikely to be or was not related to the protocol treatment. Of the initial 24 patients, 21 could thus to be used for assessment of DLT, 29 of all 32 patients for assessment of adverse events (Fig. 1).

Safety

Administration of WT1 vaccine in combination with gemcitabine was well tolerated. All adverse events are listed in Table 2. The initial assessment of safety for 21 patients found that a grade 4 central nervous system cerebrovascular ischemia considered to be a DLT had occurred in 1 patient. The most commonly reported adverse event was skin toxicity related to WT1 vaccine. All patients developed grade 1 or 2 skin reactions with swelling, redness, erythema, and induration with or without involvement of small vesicles at the local vaccine-injection sites. Hematological abnormalities were similar to those observed with the administration of gemcitabine alone, and none of the patients developed DLTs associated with hematological abnormalities or febril neutropenia. Eight grade 3 nonhematological adverse events (1 instance of hyponatremia and 7 hepatobiliary/pancreas infections) were detected and attributed to complications associated with disease progression or biliary obstruction. Other major nonhematological adverse events included grade 1 or 2 skin rash, anorexia, nausea, and fever, all of which were previously reported as major adverse events associated with gemcitabine. Hepatic transaminase elevation was principally related to disease progression and/or hepatobiliary infection. Except for local skin reactions, none of the patients experienced adverse events considered to be related to WT1 vaccination.

Clinical Response and Survival Analysis

The clinical efficacy results for all 32 patients are summarized in Table 3. Two patients were excluded from some of these analyses. One patient, who had followed a satisfactory and interesting treatment course and finally undergone a surgical resection (Supplementary Figure 1, Supplemental Digital Content 1, http://links.lww.com/JIT/A317 and Table 3), was excluded from the evaluations of response and survival because the diagnosis of pancreatic cancer could not be pathologically confirmed due to the lack of viable tumor cells in the resected specimens. The other patient was excluded from the evaluation of response because of withdrawal of consent before the first evaluation. Thus, of the total of 32 patients, 30 could be used to evaluate response to treatment and 31 to assess survival. Six of 30 patients (20.0%) reached partial response (PR), and 16 of them (53.3%) showed SD at least for >8 weeks (Table 3). Median progression-free survival was 4.2 months (95% CI, 3.6–4.6) (Fig. 2A) and MST was 8.1 months (95% CI, 6.3–10.0) (Fig. 2B). Six-month and 1-year OS rates were 71.0% (95% CI, 54.9–87.1) and 29.0% (95% CI, 12.9–45.1), respectively (Fig. 2B).

Ten of 19 patients with >100 U/mL of CA19-9 at baseline (52.6%) showed a decrease in CA19-9 serum concentration of at least 50% (Table 3).

TABLE 2. Adverse Events Reported in 29 Patients who Completed the First 2 Courses of Treatment

	Grades				N (%)		
	1	2	3	4	Any Grade (N = 29)	Grade 3 or 4 (N = 29)	DLT (N = 21)
Hematological abnormalities							
Neutropenia	3	6	13	0	22 (75.9)	13 (44.8)	0 (0.0)
Leukocytopenia	4	12	8	0	24 (82.8)	8 (27.6)	0 (0.0)
Lymphopenia	3	12	8	0	23 (79.3)	8 (27.6)	0 (0.0)
Anemia	6	15	2	0	23 (79.3)	2 (6.9)	0 (0.0)
Thrombocytopenia	15	6	1	0	22 (75.9)	1 (3.4)	0 (0.0)
Nonhematological events							
CNS ischemia	0	0	1	0	1 (3.4)	1 (3.4)	1 (4.8)
Hepatobiliary tract infection with normal ANC	0	1	7	0	8 (27.6)	7 (24.1)	0 (0.0)
Hyponatremia	3	0	1	0	4 (13.8)	1 (3.4)	0 (0.0)
Hypoalubminemia	9	4	0	0	13 (44.8)	0 (0.0)	0 (0.0)
ALT	9	4	0	0	13 (44.8)	0 (0.0)	0 (0.0)
AST	10	1	0	0	11 (37.9)	0 (0.0)	0 (0.0)
Bilirubin	2	4	0	0	6 (20.7)	0 (0.0)	0 (0.0)
Hyperkalemia	3	0	0	0	3 (10.3)	0 (0.0)	0 (0.0)
Hemorrhage in urinary tracts	2	1	0	0	3 (10.3)	0 (0.0)	0 (0.0)
Proteinuria	2	0	0	0	2 (6.9)	0 (0.0)	0 (0.0)
Hypokalemia	1	0	0	0	1 (3.4)	0 (0.0)	0 (0.0)
Anorexia	9	0	0	0	9 (31.0)	0 (0.0)	0 (0.0)
Rush*	5	3	0	0	8 (27.6)	0 (0.0)	0 (0.0)
Fever	6	1	0	0	7 (24.1)	0 (0.0)	0 (0.0)
Nausea	7	0	0	0	7 (24.1)	0 (0.0)	0 (0.0)
Diarrhea	2	1	0	0	3 (10.3)	0 (0.0)	0 (0.0)

Adverse events were graded using the National Cancer Institute Common Toxicity Criteria of Adverse Events (CTCAE version 3.0).
*Exclude skin reaction at WT1 vaccine-injection sites.
ANC indicates absolute neutrophil count; CNS, central nervous system; DLT, dose-limiting toxicity.

Correlations between CBR and either physical or functional scores assessed with the FACT-G QOL questionnaire were analyzed. For assessment of CBR, 16 of the initial 24 patients (66.7%) could be used. Nine (56.3%) of these patients (3 with PR, 5 with SD, and 1 with progressive disease) were classified as CBR responders (data not shown). CBR responders showed improvement in physical and functional scores during the first 2 courses, whereas both scores for CBR nonresponders tended to become worse (Supplementary Figure 2, Supplmental Digital Content 2, http://links.lww.com/JIT/A318).

WT1-specific Immune Response

Exploratory analyses of the immune response consisted of assessment of DTH to WT1 peptide and WT1 tetramer$^+$ CD3$^+$ CD8$^+$ T lymphocytes (WT1-CTLs) in PB of all 32 patients. All patients were DTH-negative at baseline, but 31 were at least once assessed as DTH after WT1 vaccination and 18 patients (58.1%) showed DTH-positivity, all of which conversion was detected during the protocol treatment. All of the DTH-positive patients showed at least ≥ 4 mm diameter of erythema, which was a length that was easy enough to measure. Next, for evaluation of associations between survival and DTH, the patients were classified into 4 groups according to survival time: Superior (>12 mo), good (8–12 mo), moderate (4–8 mo), and poor (≤4 mo) responders. These categories were based on the following findings: (i) MST for best supportive care only is no more than 3–4 months[1]; (ii) MST of our patients was 8.1 months; and (iii) survival time of >12 months generally indicates that the treatment has been beneficial. DTH-positivity of superior and good responders was 68.7% (11/16), whereas that of poor responders was 0% (0/7). The association between DTH-positivity and longer survival time was statistically significant ($\chi^2 = 15.908$, $P = 0.0012$) (Table 4). Therefore, survival was retrospectively reanalyzed in terms of DTH-positivity or DTH-negativity. MST was 3.9 and 10.9 months for DTH-negative (N = 13) and DTH-positive (n = 17)

TABLE 3. Summary of Clinical Efficacy Results

	All Patients	DTH Positive	DTH Negative
Best overall response [N (%)]			
Complete response	0 (0.0)	0 (0.0)	0 (0.0)
Partial response	6 (20.0)	3 (17.6)	3 (23.1)
Stable disease*	16 (53.3)	12 (70.6)	4 (30.8)
Progressive disease	8 (26.7)	2 (11.8)	6 (46.2)
Excluded	1†	1	0
Not evaluable	1		
CA19-9 response (>100 U/mL at baseline)	N = 19	N = 11	N = 7
Positive‡ [N (%)]	10 (52.6)	7 (63.6)	3 (42.9)
PFS	N = 31	N = 17	N = 13
Range (d)	21–1504 +	55–1504 +	21–373
Median PFS (mo)	4.2 (1.1–7.4)	5.4 (2.6–8.2)	2.9 (−1.6 to 7.1)
3-mo PFS (%)	67 (50–84)	82 (64–100)	46 (9–73)
OS	N = 31	N = 17	N = 13
Range (d)	30–1504 +	154–1504 +	30–443
Median OS (mo)	8.1 (6.3–10.0)	10.9 (1.2–20.7)	3.9 (−3.0–10.7)
6-mo OS (%)	71 (55–87)	88 (73–104)	46 (19–73)
12-mo OS (%)	29 (13–45)	47 (18–65)	7.7 (−6.8 to 22)

(): 95% CI.
*Stable disease conformation is determined at least for >8 weeks.
†This patient was reached partial response after 3 courses of treatment, and finally underwent the surgical resection. This patient was excluded the analysis of clinical response, PFS, and OS.
‡"Positive" CA19-9 response is defined as a ≥ 50% decrease in CA19-9 concentration after treatment.
CA19-9 indicates carbohydrate antigen 19-9; CI, confidence interval; DTH, delayed-type hypersensitivity; OS, overall survival; PFS, progression-free survival.

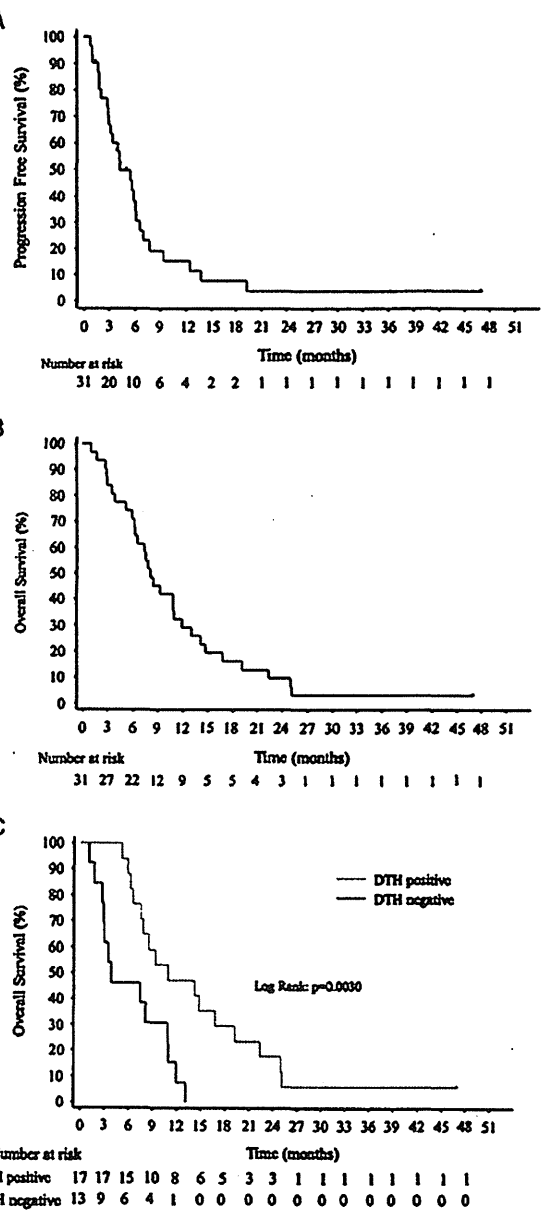

FIGURE 2. Kaplan-Meier survival curves. A, Progression-free survival (N = 31). B, Overall survival (N = 31). C, Overall survival in DTH-positive or DTH-negative patients. DTH indicates delayed-type hypersensitivity.

patients, respectively, with a statistically significant difference ($P = 0.0030$) (Fig. 2C and Table 3).

The number of WT1-CTLs and the percentages of naive ($CD45RA^+CCR7^+$), memory ($CD45RA^-CCR7^+$ and $CD45RA^-CCR7^-$), and effector ($CD45RA^+CCR7^-$) phenotypes in WT1-CTLs did not show any significant changes during the protocol treatment by the analysis using all patients (Supplementary Table 1, Supplemental Digital Content 3, http://links.lww.com/JIT/A319 and Supplementary Table 2, Supplemental Digital Content 4, http://links.lww.com/JIT/A320). Next, these immunologic parameters were compared between patients showing DTH-positivity and DTH-negativity. The difference in the number of WT1-CTLs was not statistically significant (Supplementary Table 1, Supplemental Digital Content 3, http://links.lww.com/JIT/A319). Phenotype analysis of WT1-CTLs showed that the percentage of naive-phenotype was higher in DTH-positive than in DTH-negative patients at baseline (Fig. 3A). After treatment, DTH-positive patients showed a significantly higher percentage of memory-phenotype and consequently a lower percentage of effector-phenotype WT1-CTLs than did their DTH-negative counterparts (Fig. 3A and Supplementary Table 2, Supplemental Digital Content 4, http://links.lww.com/JIT/A320). Furthermore, the percentage of memory-phenotype WT1-CTLs for the superior responders seemed to be relatively higher than that of effector-phenotype WT1-CTLs (Fig. 3B), whereas this tendency was quite the opposite for the poor responders (Fig. 3B and Supplementary Table 3, Supplemental Digital Content 5, http://links.lww.com/JIT/A321).

Case Report

A 44-year-old male with a locally advanced pancreatic head cancer (T4N1M0; stage III) received WT1 vaccine in combination with gemcitabine, and achieved PR (Fig. 4A). Five months after the beginning of the treatment, this patient underwent a complete surgical resection. Histopathologic examination of the resected specimen showed an invasive ductal adenocarcinoma with mononuclear cell infiltration around the cancer region and moderate to severe fibrotic change (Fig. 4B). This patient proved to be positive for DTH to WT1 peptide after 1 treatment course (Fig. 4C). The number of WT1-CTLs transiently decreased during the first 2–3 treatment courses but subsequently increased again, while the percentage of memory-phenotype WT1-CTLs remained high during the treatment courses (Fig. 4C). Of note, the percentage of WT1-CTLs in the tumor-infiltrating $CD3^+CD8^+$ T lymphocytes was 2.48%, which was about 6 times higher than that in PB (0.39%) (Fig. 4D). This patient had been receiving monthly administration of WT1 vaccine in combination with gemcitabine for 3 years and has maintained a Karnofsky performance status of 100% with no evidence of disease recurrence.

DISCUSSION

This study was designed with a DLT target rate of 10% during the first 2 treatment courses, but only one of the 21 initial evaluable patients (4.8%) actually experienced DLT. These results confirmed that WT1 vaccine in combination with gemcitabine is acceptable for patients with advanced pancreatic cancer. Cerebrovascular ischemia, reported here as a DLT, could be also caused by pancreatic cancer itself and/or the administration of gemcitabine, both of which are sometimes associated with a high risk of developing thrombotic disease.[27,28] Therefore, this adverse event was considered to be multifactorial and judged to be "possibly" related to treatment.

Except for skin reactions at the local injection sites, the toxicity profiles of WT1 vaccine in combination with gemcitabine were consistently similar to those of gemcitabine alone. As the *WT1* gene is physiologically expressed in hematopoietic progenitor cells,[13] damage to hematopoiesis is one of the major concerns in WT1 peptide–based immunotherapy. The incidence of hematological adverse events in our study, however, was similar to that observed for treatment with gemcitabine alone,[29] and these events were easily managed and reversible. These findings suggest that WT1 vaccine does not synergistically intensify hematological adverse events associated with gemcitabine. It seems unlikely that WT1-specific CTLs elicited by WT1 vaccine might damage normal WT1-expressing hematopoietic progenitor cells as well as WT-expressing tumor cells, as following reasons. First, in the previous clinical studies, we and others reported that WT1-specifc CTLs elicited by WT1 vaccine decreased WT1-expressing leukemia cells and suppressed the disease progression of WT-expression cancer cells, but not significantly damaged normal hematopoiesis.[19,23–26] Second, it was demonstrated that, using mice in vivo experiments, WT1-tagetting immunotherapy gave damage to tumor cells, but not WT1-expressing normal tissue, including hematopoietic cells.[40,41] The reason why the normal WT1-expressing hematopoietic cells are able to escape from the attack by WT1-specific CTLs is not well known. Further investigations should be required to address this issue.

The clinical efficacy of treatment with WT1 vaccine in combination with gemcitabine, especially in terms of survival, seemed to be better than of that with gemcitabine alone.[1,2] About half of patients who had been induced WT1-specific immunity after vaccination showed better clinical outcome with 12 months or longer survival time, suggesting additional or synergistic effects of WT1 vaccine in combination with gemcitabine. Furthermore, the former contributed to pain relief and thus to improvement of QOL. Recently, the result of the phase III study; GEST study conducted in Japan and Taiwan between 2007 and 2009 has been reported.[40] Median OS and OS rate at 12 months in the gemcitabine alone group were 8.8 months and 35.4%, respectively. These results seemed a little better than those in our study. One reason for this may be the difference in the proportion of the patients with the locally advanced pancreatic cancer, in which survival data were apparently

TABLE 4. Association Between DTH and Survival

	Overall Survival				
	>12 mo (Superior)	≤12, >8 mo (Good)	≤8, >4 mo (Moderate)	≤4 mo (Poor)	Total
DTH positive	8*	3	6	0	17*
DTH negative	1	4	1	7	13
Total	9	7	7	7	30

$\chi^2 = 15.908$, $P = 0.0012$.
*One patient was excluded from this analysis.
DTH indicates delayed-type hypersensitivity.

much better than those in metastatic ones. In our study, this proportion was 18.8%, which was lower than that in GEST study (23.8%). The other reason may be PS at baseline, which was also one of the important prognostic factors. The proportion of the patients with ECOG-PS 0, 1, and 2 at baseline in our study were 46.9%, 31.3%, and 21.9%, respectively, whereas those in GEST study were 65.3%, 34.7%, and 0.0%, respectively. It is apparent that our patients are predicted to worse prognosis than those in GEST study. Despite lower proportion of locally advanced stage and worse PS, however, the survival data gained from the patients with DTH-positivity seemed to be better than those in GEST study. These results suggested additional or synergistic effects of WT1 vaccine. Although the number of patients in our present study was too small to reach any definitive conclusions about clinical efficacy, these findings have been sufficiently encouraging to prompt us to conduct a further clinical study to determine the potency of this combination therapy. No combination chemotherapy, with the exception of FOLFIRINOX,[30] has resulted in a

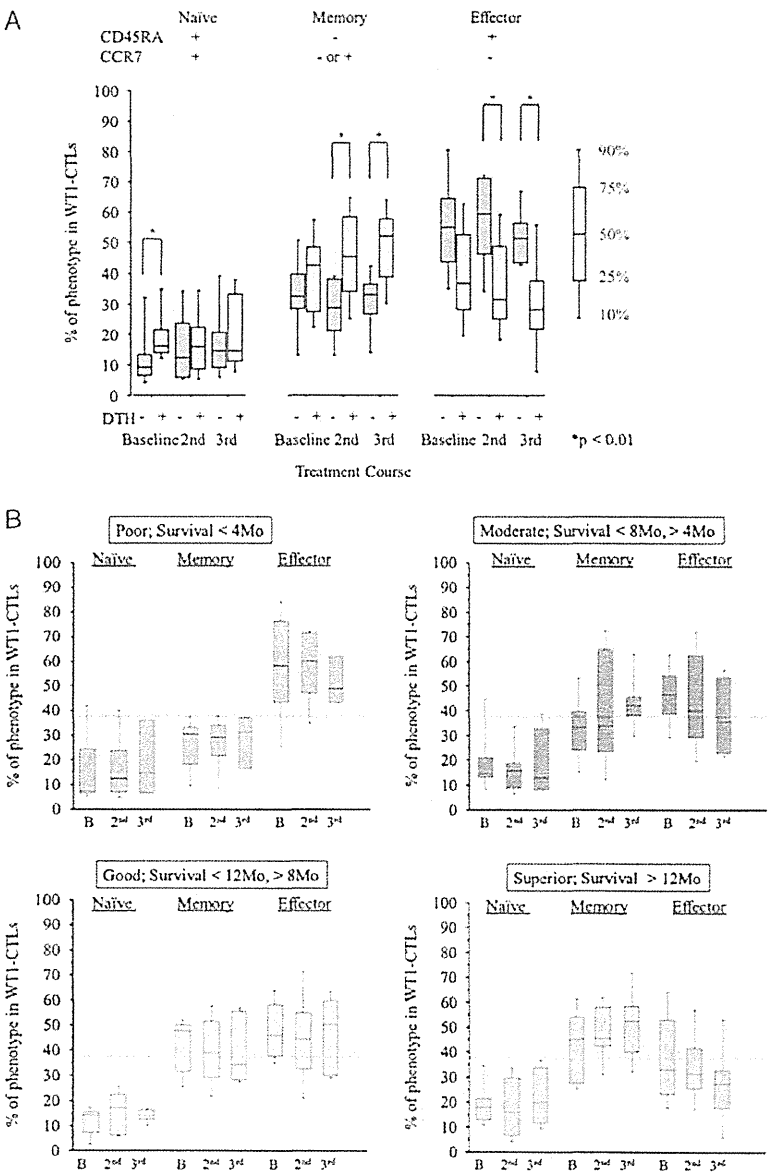

FIGURE 3. Analysis of WT1-specfic immune response. A, Immunologic monitoring of the phenotype analysis of WT1 tetramer$^+$ CD3$^+$CD8$^+$ T lymphocytes (WT1-CTLs) in DTH-positive (red columns) and DTH-negative patients (blue columns). B, Immunologic monitoring of the phenotype analysis of WT1 tetramer$^+$CD3$^+$CD8$^+$ T lymphocytes (WT1-CTLs) in the patients of 4 groups classified according to overall survival time. The broken line represents the median percentage of memory-phenotype WT1-CTLs at baseline for all patients. WT1 tetramer = PE-conjugated $WT1_{235}$ tetramer [HLA-A*24:02-restricted natural 9-mer WT1 peptide (CMTWNQMNL)], naive (CD45RA$^+$CCR7$^+$), memory (CD45RA-CCR7$^+$ or CD45RA-CCR7$^-$), and effector (CD45RA$^+$CCR7$^-$). 2nd indicates day 1 in the second course; 3rd, day 1 in the third course; B, baseline; DTH, delayed-type hypersensitivity.

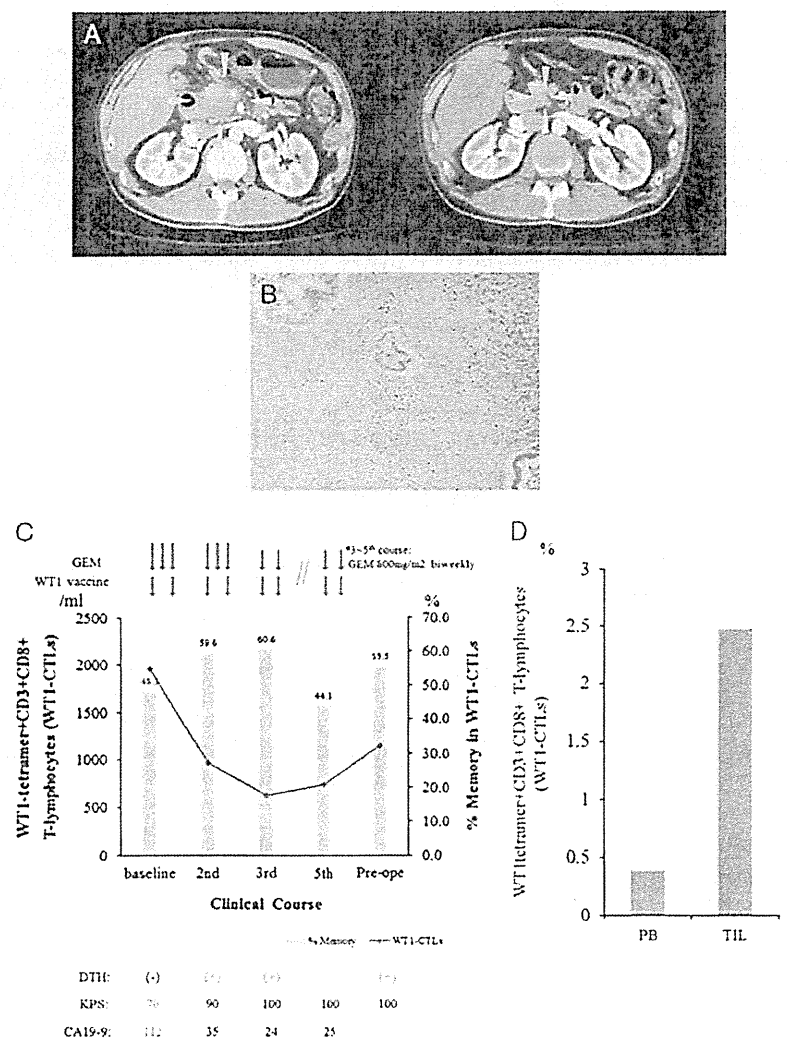

FIGURE 4. Clinical course and immunologic monitoring of 1 patient. A, Abdominal computed tomography (CT) scan before and after treatment. Left: CT scan at baseline showed a large hypodense lesion in the head of the pancreas, which had also invaded the supramesenteric artery and portal vein. Right: 5 months after treatment (before operation), a follow-up CT scan showed >80% regression of the primary lesion. Yellow arrows shows primary lesion of pancreas. B, Microscopic findings of the resected specimen (hematoxylin-eosin stain). C, Clinical course and immunologic monitoring. The blue line represents the absolute number of WT1 tetramer$^+$CD3$^+$CD8$^+$ T lymphocytes (WT1-CTLs), and the yellow column represents the percentage of memory-phenotype WT1-CTLs. D, Percentages of WT1-CTLs in the peripheral blood (PB) and tumor-infiltrating lymphocytes (TIL). CA19-9 indicates carbohydrate antigen 19-9; CTLs, cytotoxic T lymphocytes; DTH, delayed-type hypersensitivity; GEM, gemcitabine; KPS, Karnofsky performance status.

significant improvement in survival of patients with pancreatic cancer although some combination therapies are thought to be more effective for several cancers than single-agent treatments.[1] The use of FOLFIRINOX, however, may have to be limited to patients with good performance status as this regimen has much higher toxicity that sometimes can impair QOL.[31,32] In contrast, as toxicities associated with cancer vaccines are generally mild and acceptable, combination therapies using chemotherapy and cancer vaccine can be expected to exert their clinical benefits without worsening of QOL, which is often impaired by combination chemotherapies using several kinds of cytotoxic agents.

Immunologic monitoring is an important step in the development of evidence-based immunotherapy. Our data provided 2 useful prognostic markers of better clinical outcomes for the combination therapy used in our study. One is DTH to WT1 peptide and the other the frequency of memory-phenotype WT1-CTLs in PB although we did not find the correlation between clinical effects, including survival, and the frequency or absolute numbers of nonphenotypically divided WT1-specific CTLs statistically (data not shown). DTH-positive patients had a notably better prognosis than DTH-negative patients, and the OS curve for DTH-positive patients showed a late separation beyond the median. As DTH has long been used for evaluation of antigen memory for bacterial, viral, and cancer antigens,[32] the occurrence of DTH to WT1 peptide may reflect the development and persistence of memory-phenotype WT1-CTLs. This can be

inferred from our observation that DTH-positive patients showed a significantly higher frequency of memory-phenotype WT1-CTLs than did DTH-negative patients after WT1 vaccination. Furthermore, patients who survived 12 months or longer (superior responders) seemed to have the highest frequency of memory-phenotype WT1-CTLs in their PB although the number of patients in each subgroup was too small to make a statistically valid comparison. It was reported that long-term survivors who had been treated with mutant K-ras vaccine against pancreatic cancer showed the persistence of vaccinated peptide-recognizing T cells (long-term T-cell memory response) for many years after the last vaccination.[33] This report and our results suggest that the development and persistence of TAA-specific CTLs with memory-phenotype resulting from treatment with cancer vaccine contributed to the longer survival. Further investigations are needed to validate these findings in the larger-scale clinical trial.

Despite its potent cytotoxicity, gemcitabine reportedly has immune-modulating functions, such as increase in antigen cross-presentation,[34] and inhibition of B-cells,[35] myeloid-derived suppressive cells,[36] and regulatory T cells,[37] resulting in enhancement of the antigen-specific CTL function. Recently, we reported that gemcitabine enhanced the WT1 expression on human pancreatic cancer cells thus sensitizing the cancer cells to WT1-specific CTL.[11] Furthermore, it was reported that lymphopenia-induced memory-phenotype WT1-CTLs from naive-phenotype WT1-CTLs without self-antigen-induced tolerance.[38] Transient mild to moderate lymphopenia induced by gemcitabine and immediate recovery of T cells could thus promote both the differentiation of naive-phenotype WT1-CTLs into memory-phenotype WT1-CTLs and their proliferation in the clinical application of the combination therapy of gemcitabine and WT1 vaccine. In view of these immunostimulatory properties of gemcitabine, this combination therapy can be expected to generate additional or synergistic effects.

In conclusion, the combination of WT1 vaccine with the standard gemcitabine therapy was well tolerated for patients with advanced pancreatic cancer. WT1 vaccine might have additional effects on gemcitabine to improve survival benefit. An increase in memory-phenotype WT1-CTLs could be a useful predictive marker for a favorable clinical outcome. To determine the clinical efficacy of this combination therapy, we have started a phase 2 randomized clinical study (UMIN000005248).

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CONFLICTS OF INTEREST/ FINANCIAL DISCLOSURES

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All authors have declared there are no financial conflicts of interest with regard to this work.

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LETTER TO THE EDITOR

Maintenance of complete remission after allogeneic stem cell transplantation in leukemia patients treated with Wilms tumor 1 peptide vaccine

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The prognosis of patients after allogeneic hematopoietic stem cell transplantation (HSCT) is still not satisfactory because, while treatment-related mortalities have decreased, relapse after HSCT remains a major concern. The effectiveness of allogeneic HSCT for hematological malignancies is the result of immunologic rejection of recipient leukemia cells by donor T cells, known as the graft-versus-leukemia (GVL) effect.[1] It is thus obviously important to be able to exploit the GVL effect while minimizing graft-versus-host disease (GVHD). A targeted anti-leukemic immunotherapy, such as use of a leukemia vaccine,[2] is a promising strategy to boost the GVL effect.

Wilms tumor 1 (WT1) protein is one of the best targets for leukemia vaccines. Overexpression of the wild-type *WT1* gene has been detected in all types of human leukemia.[3–5] We performed a phase I clinical study of immunotherapy targeting the WT1 protein in patients with leukemia, and were able to show that WT1 vaccination was safe and could induce WT1-specific cytotoxic T lymphocyte (CTL).[6] Furthermore, reduction of minimal residual disease and long-lasting complete remission (CR) was observed in some leukemia patients who were given the WT1 vaccine.[7]

This report presents the results of phase I clinical study of WT1 vaccination for HLA-A*2402-positivie post-HSCT patients who were at high risk of relapse (HSCT in non-CR and 2nd HSCT for post-transplant relapse) or had already relapsed. The HLA-A*2402-restricted modified 9-mer WT1 peptide (amino acids 235–243 CYTWNQMNL)[8] was emulsified with Montanide ISA51 adjuvant. Patients were intradermally injected with 1.0 mg (three patients: UPNs 1, 4 and 6) or 3.0 mg (other six patients) of WT1 peptide four times weekly. When no adverse effects and no obvious disease progression were observed after the fourth injection, further WT1 vaccinations at 2-week intervals were administered.

Nine patients (five with acute myeloid leukemia (AML), one each with acute lymphoblastic leukemia, chronic myelomonocytic leukemia, multiple myeloma and T-cell lymphoblastic lymphoma) were enrolled in this study (Supplementary Tables 1 and 2). Local inflammatory response was observed at the vaccine injection sites of all patients. One patient (UPN5) suffered mild hypoxia (PaO_2 65 mm Hg at room air) and restrictive pulmonary dysfunction ($FEV_{1.0}$ 40%) 65 days after the start of WT1 vaccination (day 199 after HSCT; Figure 1a). He was diagnosed with bronchioleitis obliterans (BO), which was a symptom of chronic GVHD. The patient recovered soon after administration of inhaled steroids. While early and sudden discontinuation of prednisolone and tacrolimus (day 103 after HSCT) were considered to be the reason for development of BO, the possibility of an association between BO and WT1 vaccination cannot be entirely ruled out. In other eight patients, no severe toxicities related to WT1 vaccine were observed (Table1).

Three AML patients (UPN1–3), who had undergone HSCT in non-CR, started WT1 vaccine in CR (Supplementary Tables 1 and 2). They started WT1 vaccination on post-HSCT days 141, 76 and 93 and have remained in CR for 1038, 973 and 662 days, respectively (as of 8 April 2013; Table1), suggesting the potential of WT1 vaccination as a maintenance therapy after HSCT.

Six patients started WT1 vaccination in non-CR and two of them became CR after WT1 vaccination. One B-ALL patient (UPN4) with MLL-AF4 underwent bone marrow transplantation from an HLA-matched unrelated donor during the first CR. On post-HSCT day 111, MLL-AF4 and WT1 mRNA in peripheral blood (PB) had increased to 16 000 and 15 000 copies/μg RNA, indicating that the disease had relapsed. Tacrolimus and prednisolone doses were tapered off to induce GVL effects. The expression levels of MLL-AF4 and WT1 mRNA in PB had decreased to 2700 and 190

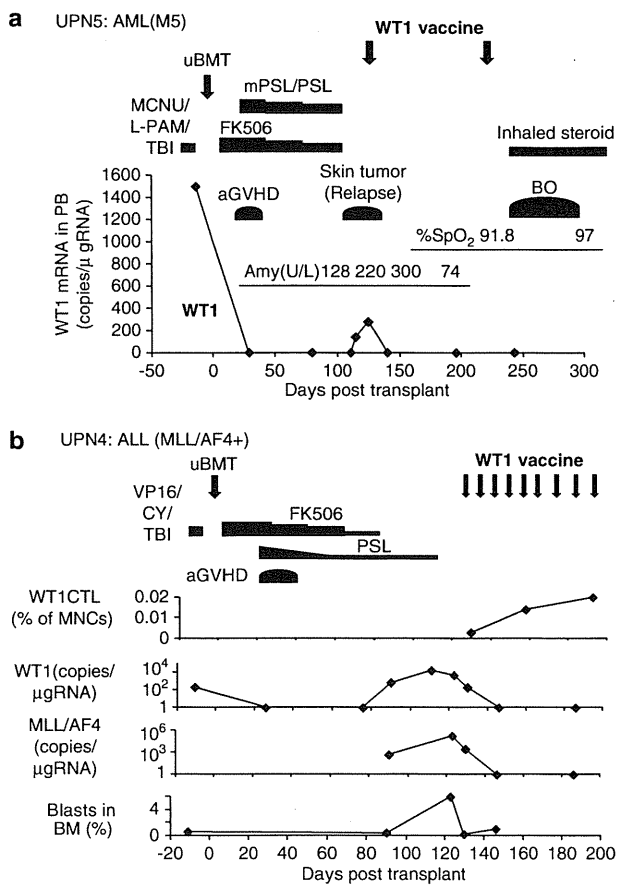

Figure 1. Clinical course of patients who attained CR after the start of WT1 peptide vaccination. (**a**) Clinical course of UPN5 who achieved CR after administration of WT1 vaccine but stopped vaccination because of the development of bronchioleitis obliterans. (**b**) Clinical course of UPN4. Residual leukemia cells that were detected by MLL/AF4 expression disappeared after the start of WT1 vaccination. In both cases, rapid tapering of immune-suppressive drugs preceded WT1 peptide vaccination.

Table 1. Patient outcomes

UPN	Disease	Status before vaccination	Adverse events	Number of vaccine doses	Outcome	Additional therapy	Survival	
							Post-HSCT	After start of vaccination
1	AML (M4)	CR	None	54	CR	–	1179+	1038+
2	AML(M4, DEK/CAN+)	CR	PLT↓	52	CR	–	1049+	973+
3	AML	CR	None	38	CR	–	759+	662+
4	B-ALL (MLL/AF4+)	Molecular relapse	None	71	CR	–	1312+	1179+
5	AML (M4)	Relapse	Amylase↑, bronchileitis obliterans (cGVHD)[a]	2	CR	–	972+	842+[b]
6	CMMoL	Relapse	None	25	PD[c]	Chemo	2265	381
7	MM	PD	None	19	PD	Chemo	1301+	804+
8	T-LBL	Relapse	None	4	PD	Second transplant	955	656
9	AML (M2)	Relapse	None	17	PD	Second transplant	1544+	749+

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CMMoL, chronic myelomonocytic leukemia; CR, complete remission; cGVHD, chronic graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; MM, multiple myeloma; PD, progressive disease; T-LBL, T-cell lymphoblastic lymphoma. (8 April 2013). [a]A causal relationship between vaccination and this event was not strongly suspected, but could not be ruled out. [b]Vaccination was discontinued. (The last injection was on post-HSCT day 60). [c]Size of the subcutaneous tumor decreased, but the disease relapsed in axial lymph nodes and stomach.

copies/μg RNA by day 132, and WT1 vaccination was started on day 133. MLL-AF4 mRNA had become undetectable by day 146, and had never appeared until post-HSCT day 1312 (day 1179 after the start of WT1 vaccination as of 8 April 2013; Figure 1b).

Skin tumors appeared in UPN5 (AML-M5) on post-HSCT day 103 and was diagnosed by biopsy as leukemia relapse. Tacrolimus was discontinued on day103, and WT1 vaccination was started on day 130. Cutaneous tumors had regressed 2 weeks after the start of WT1 vaccination, but vaccination was terminated after the second injection because of the development of BO as described earlier (Figure 1a). This patient has been remained in CR until post-HSCT day 972 (day 842 after the start of WT1 vaccination at 8 April 2013). While the exact contribution of the vaccination effect to the disease remission in addition to the GVL effect was unclear, the fact that both of these two patients still have remained in CR until now is encouraging to continue this trial. In the following phase II trials, the enumeration of WT1-specific CTLs should be performed more frequently after the start of vaccination to clarify the relationship between the effect of WT1 peptide vaccination and leukemia regression.

WT1 (a natural 9-mer WT1 peptide) HLA-A*2402 tetramer assays could be performed with peripheral blood mononuclear cell in seven of the nine patients to determine whether $WT1_{235}$ peptide-specific $CD8^+$ T cells had increased after WT1 vaccination. The gates for WT1 $tetramer^+$ cells were drawn as $<0.1\%$ of $CD8^+$ T cells were included in the tetramer-positive gate in multiple healthy individuals (Supplementary Figure 1A). $WT1_{235}$ $tetramer^+$ cells increased after the start of vaccination in three (UPNs1, 2 and 4) of the four patients who have remained in CR (Figure 1b and Supplementary Figure 1B). In the cases with progressive disease, continuous increase in the frequencies of $WT1_{235}$ $tetramer^+$ cells was not observed (Supplementary Figure 1B).

Our results suggest that WT1 vaccination should be started when the leukemia burden is minimal. The timing of the start of WT1 vaccination may be also important. For the cases with good outcomes, WT1 vaccination was started 76–140 days after transplantation (UPNs1–5), and at later times (days 299–1815) for PD cases (UPNs 6–9). A lymphopenic environment a few months after transplantation may be favorable for rapid and extensive expansion of tumor antigen-specific CTLs.

In summary, this report suggests that WT1 vaccine can be safely administrated for post-HSCT patients with hematological malignancies and has potential as a maintenance therapy. Clinical benefit of WT1 vaccination for post-HSCT patients will be evaluated in the subsequent phase II trials.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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