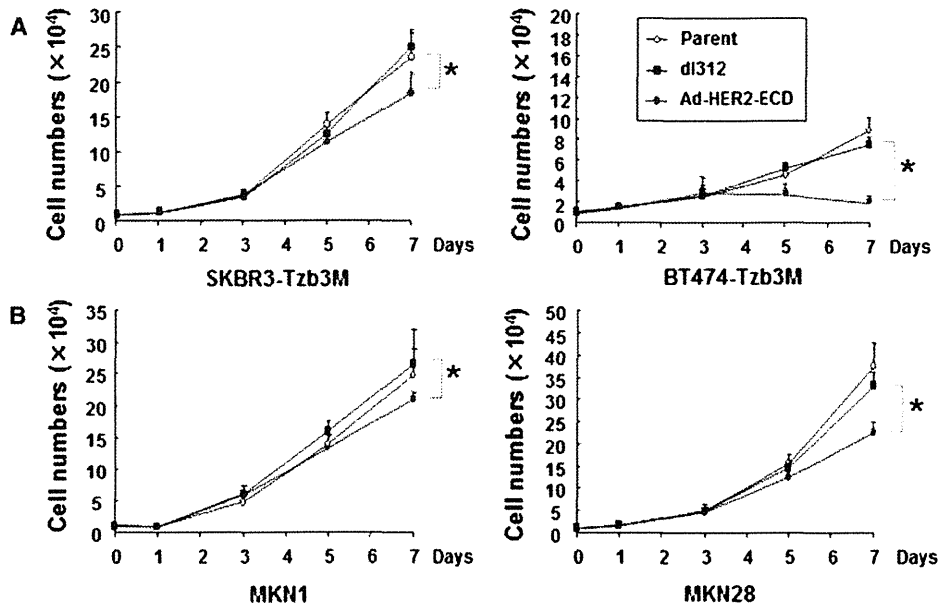

Fig. 5 Antitumor effects of Ad-HER2-ECD on trastuzumab-resistant or low HER2-expressing human cancer cells. Trastuzumab-resistant SKBR3 and BT474 breast cancer cells (**a**) and low HER2-expressing MKN1 and MKN28 gastric cancer cells (**b**) cultured as a monolayer were infected with Ad-HER2-ECD or control dl312 at an MOI of 20. The cell growth was determined by counting cell numbers each day after infection. The mean ± SD of three different wells is shown.
*$p < 0.05$

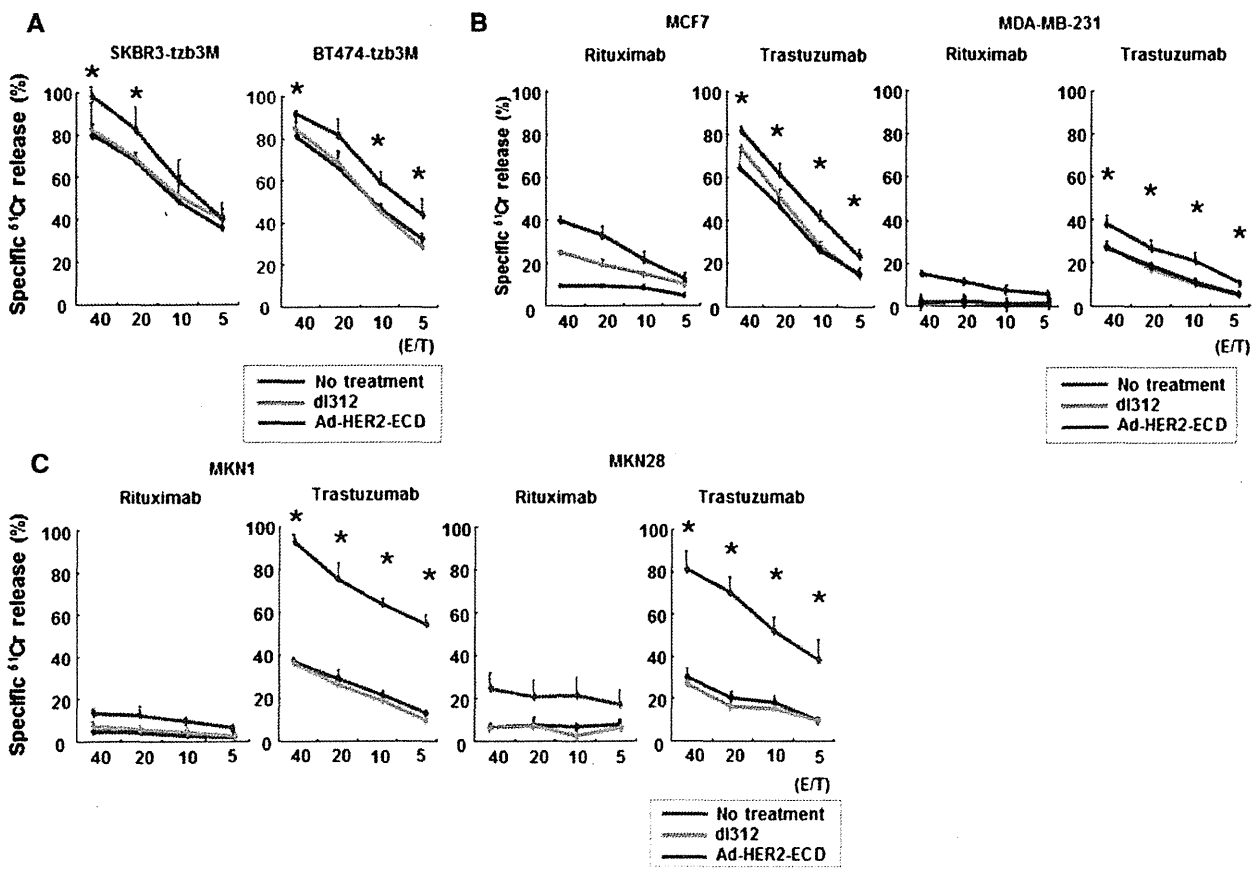

Fig. 6 Molecular sensitization of human cancer cells to trastuzumab by Ad-HER2-ECD-mediated exogenous expression of HER2-ECD. The cytotoxic reactivity of PBMCs against HER2-downregulated SKBR3 or BT474 cells (**a**), low HER2-expressing MCF7 or MDA-MB-231 human breast cancer cells (**b**), or low HER2-expressing MKN1 or MKN28 human gastric cancer cells (**c**) was assessed after Ad-HER2-ECD or dl312 infection in the presence of 10 μg/ml of trastuzumab or control rituximab by a 4-h ^{51}Cr-release assay. *Data* represent the mean ± SD of 3 wells at four different E/T ratios

responsible for this resistance. Some studies indicated that trastuzumab treatment does not alter the cell-surface HER2 expression status [30, 31]. However, we have demonstrated that continuous exposure to trastuzumab results in HER2 downregulation in HER2-overexpressing breast cancer cell lines in vitro. Previous studies also showed that alternative receptor tyrosine kinase signaling may play a role in trastuzumab resistance [18–20]. In fact, trastuzumab-exposed SKBR3 cells exhibited upregulated IGF-1R expression, suggesting that an alternative signaling pathway was enhanced to protect cells from trastuzumab-mediated HER2 signaling inhibition.

We also found that trastuzumab-exposed HER2-overexpressing breast cancer cells developed impaired trastuzumab-mediated ADCC activity in vitro. The ability of trastuzumab to mediate ADCC activity is strictly related to HER2 density [7]. In addition, Mimura et al. [32] previously reported that the HER2 status determined by flow cytometry is well correlated with trastuzumab-mediated ADCC activity in esophageal squamous cell carcinoma cell lines in vitro. Taking into account these reports, we conclude that the impaired trastuzumab-mediated ADCC activity in trastuzumab-exposed HER2-positive human cancer cells was due to the downregulation of HER2 expression on the cell surface. These results led us to examine whether exogenous expression of the HER2 receptor on the cell surface could re-sensitize HER2-downregulated human cancer cells to trastuzumab via ADCC re-activation.

HER2 overexpression is a significant prognostic factor in terms of nodal status, tumor grade, overall survival and probability of relapse in breast cancer patients [33, 34]. Although reports are conflicting, some studies have suggested that HER2-positive status in gastric cancer is associated with poor outcomes and aggressive disease [12, 13]. As expected, human cancer cells transfected with the full-length functional HER2 showed accelerated cell growth as compared to parental cells, whereas the cell growth pattern of HER2-ECD-transfected low HER2-expressing human cancer cells was similar to that of parental cells. Furthermore, we showed that HER2-ECD transfection of low HER2-expressing human cancer cells did not enhance the HER2/HER3 signaling pathway, which is the major oncogenic signal in HER2-overexpressing breast tumors [35, 36]. Although transfection of HER2-ECD-expressing plasmid did not influence cell growth, adenovirus-mediated exogenous HER2-ECD expression significantly suppressed the tumor cell growth in vitro, suggesting that the growth inhibition associated with HER2-ECD overexpression might be due to its levels on the cell surface. Therefore, Ad-HER2-ECD infection showed slightly enhanced cytotoxic activity against some types of human cancer cells even with the control antibody rituximab in the ^{51}Cr release assay. The mechanism of Ad-HER2-ECD-mediated cell growth inhibition is unclear; however, it is likely to be caused by the restriction of other HER family receptors through the formation of heterodimers with exogenously expressed HER2-ECD that lacks the downstream signaling pathway.

Some previous studies demonstrated that primary or acquired resistance to trastuzumab often results from preventing the binding of antibody to the HER2 protein by proteins such as membrane-associated glycoprotein mucin-4 [37, 38]. In our study, even after a long-term exposure to trastuzumab, trastuzumab-mediated ADCC activity on stably HER2-ECD-expressing MCF7 cells was significantly enhanced compared to mock-treated MCF7 cells, and, furthermore, HER2-downregulated or low HER2-expressing human cancer cells could be re-sensitized to trastuzumab via re-activation of trastuzumab-mediated ADCC. These results indicate that the degree of antibody-mediated ADCC activity is likely to be correlated with the cell-surface expression levels of HER2. These results suggest that the HER2-downregulated or low HER2-expressing human cancer cells exogenously overexpressing HER2-ECD is hard to develop resistance to trastuzumab in terms of the importance of ADCC activity in antitumor effects of this antibody.

A previous study has demonstrated that heterogeneity and incomplete membranous immunoreactivity for HER2 were more common in gastric cancer than in breast cancer [39], suggesting that the gastric tumors diagnosed as HER2-positive by immunohistochemistry or fluorescent in situ hybridization are more likely to be residual and regrow under trastuzumab treatment. Therefore, molecular sensitization to trastuzumab through the expression of HER2-ECD is thought to be effective even against HER2-positive gastric cancer. We would like to examine whether the ADCC activation by exogenous HER2-ECD expression functions in vivo; however, since murine NK cells do not recognize trastuzumab, which is a humanized antibody, the in vivo experiments are hard to be performed. The genetically engineered fluorescent tumor cells as well as the whole-body fluorescent imaging technology may be available for such kinds of in vivo studies [40, 41].

Although the strategy for molecular sensitization to trastuzumab via ADCC activation by using an adenoviral vector is considered to be effective, some limitations exist; for example, there are variations in the efficiency of viral infection and the expression levels of exogenous HER2-ECD. As we used a replication-deficient adenovirus vector, the viral spread might be less than ideal after intratumoral administration. We previously developed a telomerase-specific oncolytic adenovirus that causes cell death in human cancer cells with telomerase activities. These oncolytic viruses engineered to replicate in tumor cells but not in normal cells could be used as tumor-specific vectors carrying

therapeutic genes such as HER2-ECD. Moreover, ADCC activity of PBMCs from cancer patients is likely to be impaired due to immunosuppression and NK cell dysfunction, as previously reported for gastric cancer patients [42, 43]. The immunosuppressive state is associated with immunosuppressive cytokines such as IL-10 and TGF-β. These cytokines are produced within the tumor microenvironment and suppress the activity of NK cells, monocytes, and T cells [43–46]. Therefore, to sufficiently enhance the effect of trastuzumab-mediated ADCC activity in cancer patients, supportive immunotherapy such as the administration of immune-stimulating cytokines may be required.

In conclusion, our data demonstrate that HER2 downregulation and impaired ADCC activity may be one mechanism of trastuzumab resistance. We also show that exogenous overexpression of non-signaling HER2-ECD could sensitize HER2-downregulated or HER2-negative human cancer cells via ADCC activation, an outcome that has important implications for the treatment of human cancers.

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Conflict of interest All authors state that they have no potential conflicts of interest.

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Review

Ataxia-Telangiectasia Mutated and the Mre11-Rad50-NBS1 Complex: Promising Targets for Radiosensitization

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Radiotherapy plays a central part in cancer treatment, and use of radiosensitizing agents can greatly enhance this modality. Although studies have shown that several chemotherapeutic agents have the potential to increase the radiosensitivity of tumor cells, investigators have also studied a number of molecularly targeted agents as radiosensitizers in clinical trials based on reasonably promising preclinical data. Recent intense research into the DNA damage-signaling pathway revealed that ataxia-telangiectasia mutated (ATM) and the Mre11-Rad50-NBS1 (MRN) complex play central roles in DNA repair and cell cycle checkpoints and that these molecules are promising targets for radiosensitization. Researchers recently developed three ATM inhibitors (KU-55933, CGK733, and CP466722) and an MRN complex inhibitor (mirin) and showed that they have great potential as radiosensitizers of tumors in preclinical studies. Additionally, we showed that a telomerase-dependent oncolytic adenovirus that we developed (OBP-301 [telomelysin]) produces profound radiosensitizing effects by inhibiting the MRN complex via the adenoviral E1B55kDa protein. A recent Phase I trial in the United States determined that telomelysin was safe and well tolerated in humans, and this agent is about to be tested in combination with radiotherapy in a clinical trial based on intriguing preclinical data demonstrating that telomelysin and ionizing radiation can potentiate each other. In this review, we highlight the great potential of ATM and MRN complex inhibitors, including telomelysin, as radiosensitizing agents.

Key words: ATM (ataxia-telangiectasia mutated), MRN (Mre11-Rad50-NBS1) complex, radiosensitization, adenovirus, E1B55kDa

Radiotherapy is one of the standard treatment options for various malignant cancers and is often combined with surgical resection and/or chemotherapy as a part of multidisciplinary treatment. More than 50% of patients with cancer receive radiotherapy at some point during their treatment process [1]. Like surgical resection, radiotherapy is a local treatment, and it often targets not only primary tumors but also regional lymph nodes. One of the advantages of radiotherapy over surgical resection is that it is less invasive; for that reason, radiotherapy contributes significantly to treatment of cancers in areas of the body in which resection could greatly impair quality of life, such as the esophagus and the head and neck. Although the systemic side effects of radiotherapy are much less severe than those of chemotherapy, radiotherapy sometimes causes severe local adverse effects such as radiodermatitis, because normal tissues adjacent to tumors are usually included in the radiation

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fields. Although both stereotactic and fractionated radiotherapy have contributed to the improvement of irradiation methods in clinical practice, radiotherapy still has plenty of room for improvement [2, 3].

Hypoxia is one of the major limitations of radiotherapy, and researchers have made many attempts to improve it, such as through oxygenation, blood transfusion, and treatment with erythropoietin [4–6]. Although the oxygen level in a tumor is one of the most important factors in its response to radiotherapy, improving the local tumor control and survival rates for radiotherapy using pretreatment oxygenation is controversial. In one study, correction of tumor hypoxia significantly improved the locoregional tumor control and overall survival rates after radiotherapy for head and neck cancer, but was less effective for other types of cancer [7]. Although the rationale for intratumoral oxygenation before radiotherapy appears to be convincing, oxygenation alone does not improve radiotherapy sufficiently.

Many studies have been conducted in an attempt to improve radiotherapy, with much of the work being based on either of 2 hypotheses (Fig. 1). The first is that radiosensitizing agents should increase the cytotoxic effects of radiation on cancer cells by increasing the cells' radiosensitivity. The second is that radioprotective agents should decrease the adverse effects of radiation on normal cells by increasing their radioresistance. In this review, we describe several chemotherapeutic and molecularly targeted agents that have displayed radiosensitizing effects in preclinical and/or clinical studies and then focus on the potential of inhibitors of ataxia-telangiectasia (A-T) mutated (ATM) and the Mre11-Rad50-Nijmegen breakage syndrome (NBS) 1 (MRN) complex as radiosensitizing agents. Furthermore, we highlight the great potential of OBP-301 (telomelysin), a telomerase-dependent oncolytic adenovirus that we developed, as an MRN complex inhibitor.

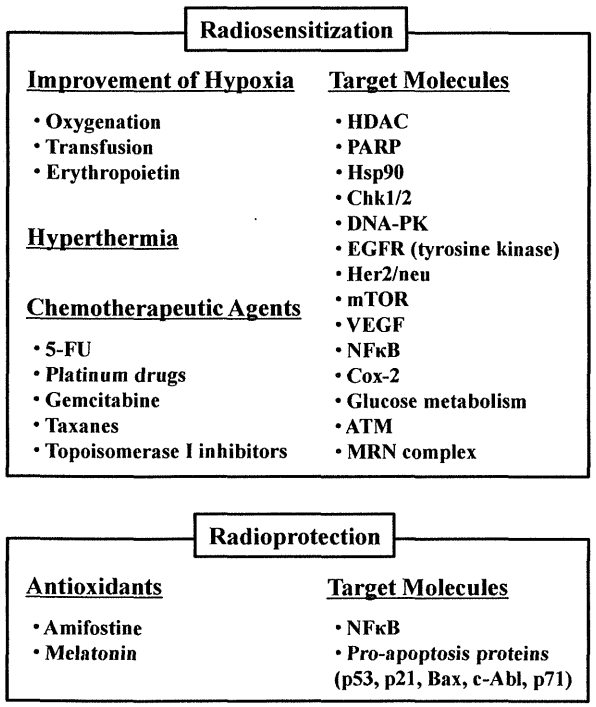

Fig. 1 Approaches to improvement of radiotherapy. Radiosensitizing agents are designed to increase the cytotoxic effects of radiation on cancer cells, and radioprotective agents are designed to decrease the adverse effects of radiation on normal cells. Hsp90, heat shock protein 90; NF-κB, nuclear factor-κB; COX-2, cyclooxygenase-2.

DNA Double-Strand Break Response: DNA Repair and Cell Cycle Checkpoints

Following DNA double strand-breaks (DSBs) induced by ionizing radiation, DNA repair and cell cycle checkpoints are the main mechanisms of maintenance of genomic stability [8]. Cells have several checkpoints that function at various phases of the cell cycle. Specifically, the G1/S and intra-S checkpoints prevent inappropriate DNA replication, whereas the G2/M checkpoint prevents cells with DNA damage from entering mitosis. When these checkpoints detect DNA damage at each phase, they induce cell cycle arrest and make time for repair of DNA damage. ATM plays a central role in the DNA damage response pathway by controlling the checkpoints via effector proteins such as Chk1, Chk2, p53 and BRCA1.

Homologous recombination (HR) and nonhomologous end joining (NHEJ) are major DNA DSB repair pathways, and cells use them according to the phase of the cell cycle and condition of the DSB ends [9, 10]. HR provides accurate genetic recombination using a sister chromatid as a template, which is essential for maintenance of genomic stability. Although HR is a desirable method of DNA DSB

repair, it is limited in cells during the S and G2 phases because of the need for a sister chromatid. NHEJ is a simple method of directly connecting the DSB ends. Although NHEJ is not as accurate as HR, it plays an important role in minimizing DNA damage, especially in cells in the G0 and G1 phases, in which HR is not available. Ku70/80, the DNA-dependent protein kinase, catalytic subunit, and DNA ligase IV are major contributors to NHEJ.

DNA repair and cell cycle checkpoints must cooperate closely to repair DNA damage and maintain genomic stability. Defects in this network produce dysfunction in the repair of DNA damage induced by ionizing radiation, which results in enhancement of the cytotoxic activity of radiation. Thus, molecules involved in these mechanisms can be suitable targets for radiosensitization.

Chemotherapeutic Agents as Radiosensitizers

As described above, radiotherapy is often combined with chemotherapy, and several chemotherapeutic agents are known to enhance the radiosensitivity of cancer cells [11, 12]. 5-Fluorouracil (5-FU), one of the most commonly used chemotherapeutic agents, is a member of the thymidylate synthase inhibitor family; these inhibitors produce cytotoxic effects by interfering with DNA synthesis [13]. Researchers have tested the combination of 5-FU and ionizing radiation and shown it to be effective against various types of cancers. This combination is a central component of current chemoradiation regimens [14].

Cisplatin, another commonly used chemotherapeutic agent, causes cytotoxicity by cross-linking DNA and interfering with cell division. Although cisplatin use is often combined with radiotherapy, oxaliplatin, another platinum derivative, has displayed more profound radiosensitizing effects [14, 15].

Gemcitabine, which is a nucleoside analogue that produces cytotoxic activity by blocking DNA replication, is another chemotherapeutic agent that is considered to be a radiosensitizer [16]. In preclinical studies, gemcitabine produced radiosensitization by interfering with Rad51 function and HR repair [17] as well as by redistributing cells into S phase by correlating with Chk1 and Chk2 [18]. Gemcitabine and radiotherapy have been shown to exert synergistic effects against cancers of the lung, pancreas, and head and neck in several clinical trials [19–21].

Taxanes such as paclitaxel and docetaxel produce cytotoxic activity by disrupting the function of microtubules that lead to cell division. A remarkable point is that taxanes arrest cells at the G2/M phase, which is the phase at which ionizing radiation is most effective [22]. Not only preclinical studies but also several clinical trials of regimens including taxanes and ionizing radiation used to treat cancers of the head and neck, esophagus, and lung have shown that taxanes are effective radiosensitizers [23–27].

Topoisomerase I inhibitors such as irinotecan, topotecan, and camptothecin interfere with topoisomerases, which are enzymes that are essential for winding and unwinding the DNA double helix during DNA replication and repair. Considering that ionizing radiation targets DNA and causes DNA DSBs, the combination of a topoisomerase I inhibitor and ionizing radiation may produce synergistic effects. Many preclinical studies using cultured cells and animal models have supported the synergy of this combination, although the specific mechanism of the synergistic effects remains unclear [28]. Also, many clinical trials have shown that these combinations are effective against various solid tumors, including head and neck, esophageal, lung, and brain tumors [29–32].

Molecularly Targeted Therapy for Radiosensitization

Although traditional chemotherapeutic agents that target rapidly dividing cells are still central to current cancer therapy, the attention of scientists is moving toward targeted therapy, which is expected to increase the effectiveness of treatment against cancer cells while reducing its harmfulness to normal cells [33]. Several small molecules and monoclonal antibodies that target epidermal growth factor receptor (EGFR), Her2/neu receptor, and vascular endothelial growth factor (VEGF) are currently in clinical use, and investigators have developed various types of molecularly targeted agents and are currently testing them in clinical trials [34, 35]. Some examples of molecularly targeted agents that are undergoing testing in clinical trials and expected to be used as radiosensitizers of tumors are described below.

Histone deacetylases (HDACs) are enzymes that control histone acetylation in coordination with the

opposing actions of histone acetyltransferases and play important roles in the regulation of gene expression. Physicians have long employed HDAC inhibitors such as valproic acid as anticonvulsants and mood-stabilizing drugs in the clinic, and use of these agents recently has generated a great deal of interest in their potential as antitumor drugs [36]. HDAC inhibitors have induced tumor-selective apoptosis and growth arrest in preclinical studies and exhibited effectiveness against tumors alone or in combination with chemotherapy in many clinical trials [37, 38]. To date, two HDAC inhibitors approved by the U.S. Food and Drug Administration—vorinostat and romidepsin—are in clinical use for treatment of T-cell lymphoma. Regarding the potential radiosensitizing effect of HDAC inhibitors, histone hyperacetylation induced by HDAC inhibitors appears to increase the cytotoxic activity of ionizing radiation [39, 40], and several clinical trials are testing these inhibitors in combination with radiotherapy for many types of cancer [41, 42].

Poly (ADP-ribose) polymerase (PARP) enzymes are proteins that play critical roles in DNA repair and replication. PARP1, which is the most abundant PARP and accounts for most PARP activities in cancer cells, binds to both DNA single-strand breaks (SSBs) and DSBs, but its role in SSB repair is better established. Although PARP inhibitors mainly contribute to SSB repair and often do not directly contribute to DSB repair, which is more critical for cell survival, defects in HR brought about by PARP inhibitors appear to increase the cytotoxic activity of ionizing radiation, especially in cells that are defective in DSB repair or NHEJ function [43–46]. Many PARP inhibitors are currently in clinical trials as single agents or in combination with DNA damage-inducing chemotherapeutic agents, and the PARP inhibitor ABK-888 administered in combination with radiotherapy recently entered clinical trials [47].

In addition, inhibitors of heat shock protein 90 or Chk1/2, some of which are currently in clinical trials as monotherapy or in combination with chemotherapeutic agents, have exhibited potential as radiosensitizers in preclinical studies, although combinations of them with radiotherapy have yet to be tested in clinical trials as far as we know [48–50]. Some EGFR tyrosine kinase inhibitors such as erlotinib and gefitinib and VEGF inhibitors such as bevacizumab, which are currently in clinical use for cancer therapy, also have displayed radiosensitizing effects in many preclinical studies and clinical trials [51].

ATM as a Target for Radiosensitization

As described above, molecules involved in DNA repair or cell cycle checkpoints can be targets to enhance tumor radiosensitivity. Interest in molecularly targeted therapy has deepened our understanding of the signaling pathways for DNA repair and cell cycle checkpoints, and ATM has been revealed to play a central in these signaling pathways. Studies originally identified the *ATM* gene in A-T, a disease that causes several severe disabilities, such as cerebellar degeneration, immunodeficiency, hypersensitivity to radiation and genomic instability, and increased incidence of malignancies [52, 53]. All patients with A-T have mutations in the *ATM* gene, and intensive investigation of such patients and A-T cells has contributed to the elucidation of ATM function. The construction of the ATM protein is similar to that of ATM- and RAD3-related (ATR), the DNA-dependent protein kinase, catalytic subunit, and mammalian target of rapamycin (mTOR), and ATM belongs to the phosphatidylinositol 3-kinase (PI3K)-related kinase family.

Following DNA damage, ATM immediately activates signaling pathways for DNA repair and cell cycle checkpoints. Although recent studies have shown that downstream signaling of ATM is becoming increasingly complicated, p53 and Chk2 are undoubtedly the main targets of ATM and control the G1/S and G2/M checkpoints while interacting with each other. Also, inhibition of these checkpoints allows damaged cells to move to the mitotic phase without undergoing proper DNA repair, leading to mitotic catastrophe, which is currently considered a main cause of cell death induced by radiotherapy [54–56]. Moreover, ATM is known to affect HR repair by directly or indirectly phosphorylating at least 12 targets, such as BRCA1/2 and NBS1, and defects in ATM function lead to dysfunction in HR repair [57, 58]. These findings indicate that targeted ATM inhibition is an attractive approach to enhancing tumor radiosensitivity.

Caffeine and wortmannin, which are nonspecific PI3K inhibitors, have been widely used in studies related to ATM/ATR functions [59, 60]. However,

some of the effects of caffeine and wortmannin in cells, such as apoptosis and checkpoint abrogation, are caused not only by ATM/ATR inhibition but also by other factors in the PI3K family [60, 61]. Recently, researchers developed several more specific ATM and ATM/ATR inhibitors—KU-55933, CGK733, and CP466722—and tested their potential as radiosensitizers in preclinical studies. KU-55933 was found to exhibit a specific inhibitory effect on ATM but not on other PI3K-family proteins, such as PI3K, DNA-PK, ATR, and mTOR, and sensitized cells to ionizing radiation by blocking phosphorylation of γH2AX, NBS1, and Chk1 [62]. CGK733 demonstrated selective inhibition of ATM and ATR, which led to blockage of the checkpoint signaling pathways, and researchers showed that its inhibitory effects were more beneficial than its small interfering RNA-mediated inhibition [63]. CP466722 exhibited inhibition of ATM and its downstream signaling pathways in the same way that KU-55933 did, and investigators emphasized that transient (4h or less) inhibition of ATM expression was sufficient to increase the radiosensitivity of tumor cells [64]. Small interfering RNAs and antisense DNA for ATM also exhibited potent radiosensitizing effects [65, 66]. Based on this preclinical evidence, ATM inhibitors are expected to be promising candidate radiosensitizers.

The MRN Complex as a Target for Radiosensitization

Although the importance of the ATM signaling pathway in DNA repair and cell cycle checkpoints has been established, the MRN complex has emerged as an essential factor in ATM activation. Mre11 and Rad50 were originally isolated from the yeast *Saccharomyces cerevisiae* in genetic screens in which an Mre11 mutant was defective in meiotic recombination [67] and a Rad50 mutant was sensitive to DNA damage [68]. NBS1 was isolated as a member of the complex that binds with Mre11 and Rad50, and mutations in this gene cause NBS, which is characterized by high cancer incidence, cell-cycle-checkpoint defects, and radiosensitivity [69]. Mutations in the *Mre11* gene have been reported to cause A-T like disorder [70], and deficiency of the *Rad50* gene causes NBS-like disorder [71]. The indispensability of the MRN complex to cells is emphasized by the fact that null mutations of either of these genes cause embryonic lethality in mice [72]. The Mre11 protein is uniformly distributed in the nucleus under undamaged conditions, but it migrates to sites of damage within 30 minutes after DNA DSB induction and forms a complex with Rad50 and NBS1, which is visualized as nuclear foci [73].

The MRN complex plays important roles in signal transduction related to DNA repair and cell cycle checkpoints [10]. One of these roles is activation of the ATM/ATR signaling pathway. Dysfunction of the MRN complex results in impairment of the ATM signaling pathway, which leads to hypersensitivity to DNA-damaging agents. The MRN complex has also been reported to contribute to the DNA DSB-repair pathway directly or indirectly via ATM activation [9]. In the HR repair process, the MRN complex serves as a primary damage sensor and is involved in the early steps of HR repair, which include processing of the broken DNA ends: in other words, removal of the 5' strand to uncover the 3' single strand [74]. Whereas Ku70/80 and DNA-PK are well known to be the main components in NHEJ, the importance of the MRN complex to NHEJ has only recently been demonstrated, and whether the MRN complex is correlated with Ku70/80 and DNA-PK in NHEJ remains unclear [10, 75].

As might be expected from the fact that mutations in members of the MRN complex are hypersensitive to DNA DSBs, inhibitors of the MRN complex enhance the cytotoxic activity of ionizing radiation. Although disruptions of the MRN complex by gene therapy have been reported to be effective in combination with radiotherapy, researchers recently isolated a novel small-molecule inhibitor of the MRN complex called mirin from a chemical genetic screen [76, 77]. Mirin inhibited MRN complex-dependent ATM activation and Mre11-associated exonuclease activity, leading to abolishment of the G2/M checkpoint and impairment of HR repair. These results are consistent with the known and anticipated functions of the MRN complex. Considering the importance of the MRN complex in DNA repair and cell cycle checkpoints, MRN complex inhibitors appear to be very promising as radiosensitizers.

The Radiosensitizing Effect of the Adenoviral E1B55kDa Protein

We recently demonstrated that telomelysin sensitizes cancer cells to the cytotoxic activity of ionizing radiation [78]. Telomelysin is a telomerase-dependent oncolytic adenoviral agent whose replication is controlled by the human telomerase reverse transcriptase (hTERT) promoter. Telomelysin can thus induce cell death via oncolysis by replicating only in cancer cells whose hTERT activity is high [79–81]. An American Phase I clinical trial of single-agent telomelysin evaluated the clinical safety and pharmacokinetics of the agent in the human body following its approval by the U.S. Food and Drug Administration in 2006. When injected intratumorally in patients with various solid tumors such as melanoma, sarcoma, lung cancer, breast cancer, and head and neck cancer, telomelysin proved to be effective and well-tolerated without any severe adverse events [82].

The adenoviral E1B55kDa protein has been reported to play an important role in creating the optimal intracellular environment for adenoviral protein synthesis by inhibiting the function of the MRN complex and p53 in cooperation with the adenoviral E4 protein [83]. Inhibition of the MRN complex is also considered to be a self-defense response to concatemer formation of the double-strand DNA genome of adenovirus by the MRN complex [84–86]. We showed that expression of the MRN complex in cancer cells began to decrease about 24 h after telomelysin treatment, when the E1B55kDa protein began to be expressed, which led to inhibition of ATM phosphorylation by ionizing radiation and inhibition of DNA repair. We determined the importance of the presence of E1B55kDa in regard to this inhibitory effect by comparing telomelysin with the E1B-defective oncolytic adenovirus dl1520 (onyx-015), which has been used in many clinical trials [87].

We demonstrated that inhibition of the MRN complex by telomelysin via the E1B55kDa protein produced a profound radiosensitizing effect *in vitro*; interestingly, on the other hand, ionizing radiation increased the cytotoxic activity of telomelysin, presumably by increasing viral uptake into cancer cells, which means that telomelysin and ionizing radiation potentiate each other. Furthermore, combined therapy with telomelysin and ionizing radiation exhibited a strong synergistic antitumor effect in animal studies [78]. A clinical study of the combination of telomelysin and ionizing radiation against cancers of the head and neck and esophagus is currently under consideration in Japan, and additional telomelysin-based treatment is expected to contribute to improvement of the survival rates and quality of life in patients with these cancers. Moreover, this inhibitory effect on the MRN complex via the E1B55kDa protein may apply to not only telomelysin but also all of the other oncolytic adenoviruses that produce this protein, which may provide new clues to clinical applications of oncolytic adenovirotherapy (Fig. 2).

Perspectives on ATM and MRN Complex Inhibitors

Precise cellular responses to DNA DSBs require efficient recognition of the damaged DNA sites and organized activation of the signaling pathways leading to DNA repair and cell cycle checkpoints. Numerous preclinical studies have shown that ATM and the MRN complex play critical roles in this response, which indicates that these molecules are promising targets for radiosensitization. In fact, the ATM and MRN complex inhibitors described above have exhibited profound radiosensitizing effects in preclinical studies. The next step should be to test these inhibitors toward clinical application is to be tested in clinical settings, but to our knowledge, none of them have entered clinical trials.

One of the factors that could impede the success of ATM and MRN complex inhibitors in clinical trials is tumor selectivity. The expression and functions of ATM and the MRN complex do not appear to differ much in cancer cells and normal cells, which means that unless these inhibitors are delivered to tumors selectively, severe adverse events may occur when they are combined with radiotherapy. Recent developments in the field of drug delivery could have remarkable outcomes when combined with developments in the field of drug discovery. For example, nanomedicine has revolutionized drug delivery, and nanosized carriers such as liposomes, polymers, and micelles increase the stability of therapeutic drugs in the bloodstream [88]. Moreover, these carriers can acquire tumor-targeting potential by being equipped with antibodies or peptides that target biomarkers that are overex-

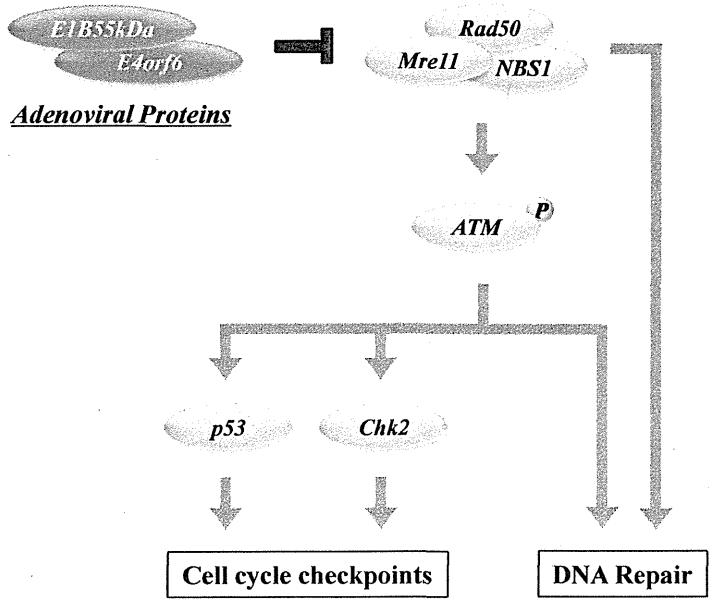

Fig. 2 The molecular mechanism of radiosensitization via the adenoviral E1B55kDa protein. E1B55kDa inhibits the function of the MRN complex in cooperation with the adenoviral E4orf6 protein, which inhibits the ATM signaling pathway and leads to cell-cycle-checkpoint abrogation and DNA-repair dysfunction.

pressed in tumors [89]. This type of improvement in drug delivery may be necessary for the use of ATM or MRN complex inhibitors before they enter clinical trials.

Regarding tumor-targeting potential, telomelysin may be a step ahead of these ATM or MRN complex inhibitors because its effect is strictly limited to cancer cells with high telomerase activity levels. Moreover, Phase I clinical trials in the United States have already determined the safety of monotherapy with telomelysin, and this agent is about to undergo testing in combination with ionizing radiation in a clinical trial in Japan.

However, telomelysin also has some challenging drawbacks that must be overcome in order to increase its attractiveness and its application as a cancer therapeutic agent. One of these issues is that telomelysin currently can only be administered via local injection and not systemically. The majority of intravenously administered adenoviruses become trapped in the liver, and thus they are not present at sufficient levels at the tumor sites [90]. In addition, most people have neutralizing antibodies against adenovirus type 5, which is one of the common cold viruses. Therefore, telomelysin, which consists of this adenovirus, is removed by the immune system immediately after systemic administration. For this reason, application of telomelysin is currently limited to cancers confined within locoregional areas, and improvements in telomelysin that would facilitate its systemic delivery will be needed before the drug can be used in the treatment of distant metastases.

In summary, the field of targeted radiosensitization of tumors is developing rapidly and drawing much attention. ATM and the MRN complex play central roles in the DNA DSB-response pathways, and inhibitors of these molecules are promising candidate radiosensitizing agents. An upcoming clinical trial of telomelysin combined with ionizing radiation will test this agent's function as an MRN complex inhibitor, and the outcome of this trial is expected to open new opportunities for other oncolytic adenoviruses that produce the E1B55kDa protein as promising radiosensitizers.

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Biased usage of T cell receptor β-chain variable region genes of Wilms' tumor gene (WT1)-specific CD8$^+$ T cells in patients with solid tumors and healthy donors

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Wilms' tumor gene 1 (WT1) protein is a promising tumor-associated antigen. In patients with WT1-expressing malignancies, WT1-specific CTLs are spontaneously induced as a result of an immune response to the WT1 protein. In the present study, we performed single cell-level comparative analysis of T cell receptor β-chain variable region (TCR-BV) gene families of a total of 750 spontaneously induced $WT1_{126}$ peptide (amino acids 126–134, $WT1_{126}$)-specific CTLs in both HLA-A*0201$^+$ patients with solid tumors and healthy donors (HDs). This is the first report of direct usage analysis of 24 kinds of TCR-BV gene families of $WT1_{126}$-specific CTLs at the single cell level. Usage analysis with single-cell RT-PCR of TCR-BV gene families of individual FACS-sorted $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells showed, for the first time, that: (i) BVs 3, 6, 7, 20, 27, and 28 were commonly biased in patients and HDs; (ii) BVs 2, 11, and 15 were biased only in patients; and (iii) BVs 4, 5, 9, and 19 were biased only in HDs. However, statistical analysis of similarity of individual usage frequencies of 24 kinds of TCR-BV gene families between patients and HDs indicated that the usage frequencies of TCR-BV gene families in patients reflected those in HDs. These results should provide us with a novel insight for a better understanding of WT1-specific immune responses. (*Cancer Sci* 2012; 103: 408–414)

Wilms' tumor gene (*WT1*) encodes a zinc-finger transcription factor and plays important roles in the regulation of cell proliferation, differentiation, and apoptosis.[1–3] The *WT1* gene was originally isolated as the gene responsible for a childhood renal neoplasm, namely Wilms' tumor, and was first categorized as a tumor-suppressor gene.[4,5] However, based on the result of a series of studies,[6–8] we proposed that the wild-type *WT1* gene had an oncogenic rather than a tumor-suppressor function in various kinds of hematological malignancies and solid tumors. Indeed, the *WT1* gene is expressed at high levels in acute myeloid leukemia (AML), acute lymphocytic leukemia, chronic myelogenous leukemia, and myelodysplastic syndromes (MDS), as well as in various types of solid tumors.[9–14] Because a correlation has been shown between *WT1* mRNA transcript levels and the amount of minimal residual disease (MRD) in the peripheral blood (PB) or bone marrow of leukemia patients,[15–17] measurement of *WT1* mRNA transcripts is now being used to monitor MRD in leukemia patients.

Previous studies have reported that WT1-specific CTLs can be generated from human PBMC in a human leukocyte antigen (HLA) Class I-restricted manner and can lyse WT1-expressing tumor cells as well as WT1 peptide-pulsed target cells.[18,19] Mice immunized with WT1 peptide or WT1 plasmid DNA elicit WT1-specific CTLs and reject challenges by WT1-expressing tumor cells.[20,21] Furthermore, WT1-specific CTLs and antibodies are induced spontaneously in WT1-expressing tumor-bearing patients.[22–24] These results indicate that the WT1 protein is highly immunogenic and a promising target antigen for cancer immunotherapy. In fact, WT1 has been rated as the most promising cancer antigen of 75 tumor-associated antigens.[25]

On the basis of the results of these preclinical studies, clinical studies of WT1 peptide vaccination were undertaken,[26–29] with promising clinical effects, including a reduction in leukemic blast cells and tumor size, as well as long-term stable disease, being seen in association with an increase in the frequency of WT1-specific CD8$^+$ T cells in PB.[26,27] In this context, analysis of the clonality of the WT1-specific CTLs is important to gain a better understanding of the WT1-specific CTL response in WT1-expressing tumor-bearing patients and, further, to obtain clues as to how to enhance WT1-specific CTL responses in WT1 immunotherapy.

Recently, using single-cell RT-PCR analysis of the T cell receptor β-chain variable region (TCR-BV) genes of individual FACS-sorted WT1 tetramer$^+$ CD8$^+$ T cells, we demonstrated biased usage of TCR-BV gene families of $WT1_{235}$ peptide (amino acids 235–243)-specific CTLs in HLA-A*2402$^+$ patients with AML or MDS, which reflected the biased usage in healthy donors (HDs).[30]

In the present study, we examined usage frequencies of TCR-BV gene families of CTLs specific for $WT1_{126}$, an HLA-A*0201-restricted CTL epitope, in both patients with solid tumors and HDs and found biased usage for these TCR-BV gene families in both the patients and HDs and that the patterns of biased usage were very similar between the two groups.

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Table 1. Characteristics of the patients and healthy donors

	Gender	Age (years)	Disease	Clinical stage	Prior therapy
Patients					
PT-1	M	33	GBM	N/A	Ope/RT
PT-2	F	56	GBM	N/A	Ope/RT
PT-3	M	28	GBM	N/A	Ope/RT/Chemo
PT-4	M	18	PNET	IV	Ope/RT/Chemo/ auto-PBSCT
PT-5	F	53	Ovarian cancer	IIIc	Ope/Chemo
PT-6	F	73	Cecal cancer	IV	Ope/Chemo
Healthy donors					
HD-1	F	23			
HD-2	M	45			
HD-3	F	24			
HD-4	F	25			
HD-5	M	37			

auto-PBSCT, autologous peripheral blood stem cell transplantation; Chemo, chemotherapy; GBM, glioblastoma multiforme; N/A, not available; Ope, operation; PNET, primitive neuroectodermal tumor; RT, radiation therapy.

Materials and Methods

Samples of PB from patients with solid tumors and HDs. Analysis of $WT1_{126}$-specific CTLs in PBMC was approved by the Institutional Review Board for Clinical Research, Osaka University Hospital. After written informed consent had been obtained, PB samples were obtained from six HLA-A*0201$^+$ patients with a solid tumor (patient (PT)-1, -2, -3, -4, -5, and -6) and five HLA-A*0201$^+$ HDs. Expression of WT1 protein in tumor cells was determined by immunohistochemical analysis, as described elsewhere.[31] The PBMC were separated by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use. Table 1 summarizes the characteristics of both the patients and HDs.

Flow cytometric analysis and single-cell sorting of WT1 tetramer$^+$ CD8$^+$ T cells. Thawed PBMC were rested at 37°C for 1.5 h in RPMI 1640 containing 10% FBS before being stained with phycoerythrin (PE)-labeled HLA-A*0201/$WT1_{126}$ tetramer ($WT1_{126}$ tetramer; MBL, Tokyo, Japan) in FACS buffer composed of PBS containing 5% FBS at 37°C for 30 min. The PBMC were then stained with a panel of mAbs at 4°C for 25 min in the dark, washed three times with FACS buffer, and finally resuspended in appropriate quantities of FACS buffer. The following mAbs were used: anti-CD4-FITC, anti-CD16-FITC, anti-CD45RA-allophycocyanin (APC) (BioLegend, San

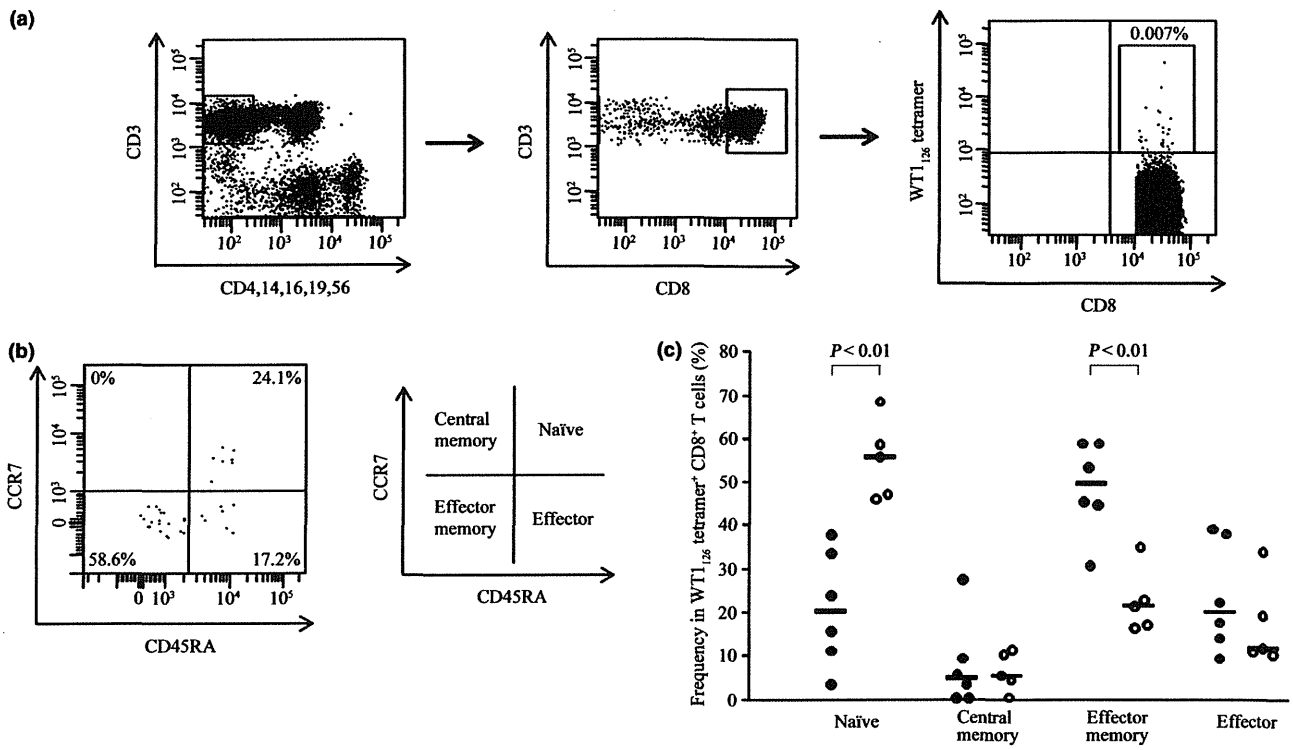

Fig. 1. Frequencies of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells in peripheral blood of patients with a solid tumor and healthy donors and phenotypic analysis of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. (a) Representative data of flow cytometric analysis using $WT1_{126}$ tetramer. CD4$^-$, CD14$^-$, CD16$^-$, CD19$^-$, CD56$^-$, and $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells were defined as $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. The percentages shown represent the frequencies of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells among total CD3$^+$ CD8$^+$ T cells. (b) $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells were classified into four distinct differentiation stages according to the cell surface expression of CCR7 and CD45RA as follows: (i) CCR7$^+$ CD45RA$^+$ (naïve) cells; (ii) CCR7$^+$ CD45RA$^-$ (central memory) cells; (iii) CCR7$^-$ CD45RA$^-$ (effector memory) cells; and (iv) CCR7$^-$ CD45RA$^+$ (effector) cells. Representative data from Patient 3 are shown. (c) Frequencies of each subset of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. Closed and open circles represent patients and healthy donors, respectively. Bars indicate the median values of the frequencies.

Diego, CA, USA); anti-CD19-FITC, anti-CCR7-PE-Cy7 (BD Pharmingen, San Diego, CA, USA); anti-CD3-peridinin chlorophyll protein (PerCP), anti-CD8-APC-Cy7, anti-CD14-FITC (BD Biosciences, San Jose, CA, USA); and anti-CD56-FITC (eBioscience, San Diego, CA, USA). In the present study, lineage antigen (CD4, CD14, CD16, CD19, and CD56)-negative, CD3-, CD8-, and $WT1_{126}$ tetramer-positive lymphocytes were defined as $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. The $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells were single-cell sorted using a FACSAria instrument (BD Biosciences), and data were analyzed using FACSDiva software (BD Biosciences).

Synthesis of cDNA from a single cell-sorted $WT1_{126}$ tetramer$^+$ CD8$^+$ T cell and determination of TCR-BV gene families. The $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells were directly single-cell sorted into PCR tubes containing cDNA reaction mix, and cDNA synthesis was performed as described previously.[30] The cDNA was amplified using 24 kinds of TCR-BV gene family-specific forward primers and a constant region-specific reverse primer.[30] Next, the PCR products were amplified by semi-nested PCR for the screening of the BV gene family as follows: the first PCR product was put into eight separate tubes, each of which was filled with a reaction mix containing the reagents, one of eight kinds of screening sets of BV gene family-specific forward primers and the reverse primer. The eight kinds of screening sets used in the present study were the same as those used in a previous study.[30] Each screening PCR product was run on a 2% agarose gel to identify the positive reaction among the eight kinds of screening sets. Finally, the TCR-BV gene family was identified by the second round of PCR using an individual TCR-BV gene family-specific forward primer, which was contained in the positive screening set, and the reverse primer. As a negative control, three PCR tubes without sorted cells were prepared in each experiment and were subjected to the same RT-PCR procedures.

A total of 750 $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells were analyzed in six patients (i.e. 59, 66, 46, 67, 88, and 73 cells from PT-1, -2, -3, -4, -5, and -6, respectively) and five HDs (i.e. 53, 57, 77, 79, and 85 cells from HD-1, -2, -3, -4, and -5, respectively). The International Immunogenetics Information System (IMGT) database site (http://www.imgt.org/IMGT_vquest/vquest?livret=0&Option=humanTcR, accessed 15 Nov 2011) was used to identify the human TCR-BV gene family.

Statistical analysis. The Mann–Whitney *U*-test was used to evaluate differences in frequencies and subset compositions of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells and CD3$^+$ CD8$^+$ T cells between patients and HDs. The significance of differences in usage frequencies of the 24 kinds of BV gene families between patients and HDs was also assessed using the Mann–Whitney *U*-test. Analyses were performed with the Stat Flex statistical software package (Artech, Osaka, Japan).

Results

Increase in $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells with effector memory phenotype in HLA-A*0201$^+$ patients with solid tumors. The CTL responses to an HLA-A*0201-restricted epitope $WT1_{126}$ of the WT1 protein were examined in HLA-A*0201$^+$ patients with solid tumors. The PBMC were FACS analyzed by using $WT1_{126}$ tetramer (Fig. 1), with Figure 1(a) showing representative profiles of the FACS analysis of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. The frequencies of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells in patients and HDs were 0.007–0.122% (median 0.039%) and 0.009–0.079% (median 0.016%), respectively. Although there was a tendency for higher frequencies in patients than in HDs, the differences failed to reach statistical significance (data not shown).

Human CD3$^+$ CD8$^+$ T cells can be divided into four distinct differentiation stages according to the cell surface expression of CCR7 and CD45RA as follows: (i) CCR7$^+$ CD45RA$^+$ (naïve) cells; (ii) CCR7$^+$ CD45RA$^-$ (central memory) cells; (iii) CCR7$^-$ CD45RA$^-$ (effector memory) cells; and (iv) CCR7$^-$ CD45RA$^+$ (effector) cells.[32,33] These cell surface markers were used to classify the differentiation stages of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells and a representative pattern from PT-3 is shown in Figure 1(b). The frequency of the naïve phenotype of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells was significantly higher in HDs than in patients (45.8–68.4% [median 55.6%] *vs* 3.4–37.9% [median 19.9%], respectively; $P < 0.01$), while the frequency of the effector memory phenotype of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells was significantly higher in patients than in HDs (30.3–58.6% [median 49.0%] *vs* 15.8–34.4% [median 20.7%], respectively; $P < 0.01$; Fig. 1c). In contrast, there were no significant differences in frequencies of the four subsets of the whole CD3$^+$ CD8$^+$ T cells between patients and HDs (data not shown),

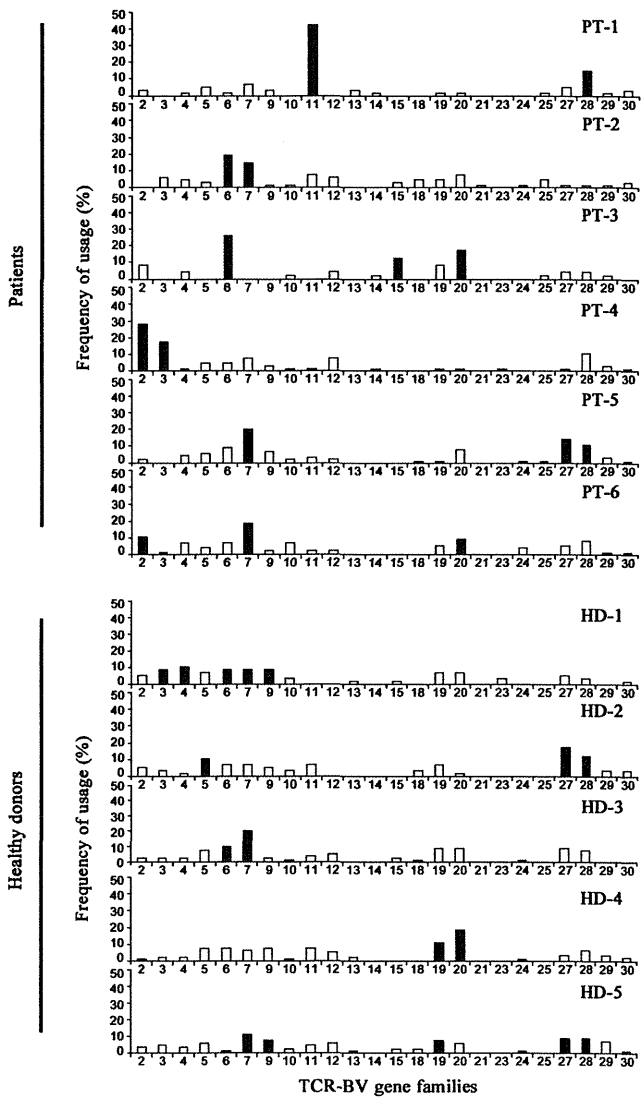

Fig. 2. Frequencies of T cell receptor β-chain variable region (TCR-BV) gene families used by T cell receptors (TCRs) in $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. The usage frequencies were defined as the ratio of (the number of a given TCR-BV gene family used)/(the total number of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells analyzed). Closed columns indicate that the usage frequency is higher than the mean value + 1SD.

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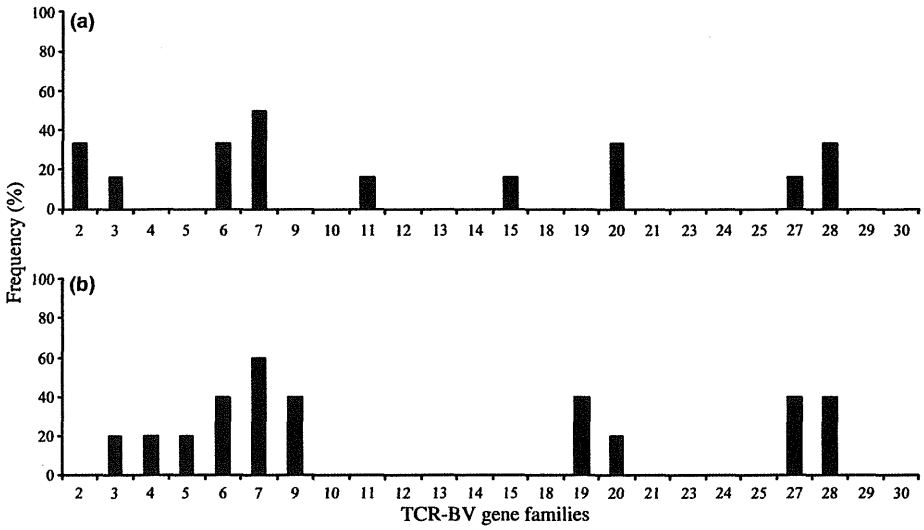

Fig. 3. Usage frequencies of T cell receptor β-chain variable region (TCR-BV) gene families with biased usage in (a) patients and (b) healthy donors. The ratios show the number of patients or healthy donors with biased usage of the specific TCR-BV gene families to the total number of patients or healthy donors examined, respectively.

indicating that the phenotypic difference in $CD3^+$ $CD8^+$ T cells between patients and HDs was restricted to $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells. These results demonstrate that $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells exhibit more differentiated/activated phenotypes in patients than in HDs.

Biased usage of TCR-BV gene families in $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells. In the present study, TCR-BV gene families in $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells were investigated by using the single cell-based RT-PCR technique for the six patients and five HDs. Usage frequencies for a given BV gene family were defined as the ratio of the number of $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells with the usage of the BV gene family to the total number of $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells analyzed. When the usage frequency of a given BV gene family was more than the mean value + 1SD for the usage of 24 different kinds of BV gene families, the usage was defined as biased, as described previously.[30] As shown in Figure 2, the biased usage of the TCR-BV gene families was as follows: BV2, in two of six patients; BV3, in one of six patients and one of five HDs; BV4, in one of five HDs; BV5, in one of five HDs; BV6, in two of six patients and two of five HDs; BV7, in three of six patients and three of five HDs; BV9, in two of five HDs; BV11, in one of six patients; BV15, in one of six patients; BV19, in two of five HDs; BV20, in two of six patients and one of five HDs; BV27, in one of six patients and two of five HDs; and BV28, in two of six patients and two of five HDs.

The ratios of the number of patients or HDs with biased usage of individual TCR-BV gene families in $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells to the number of patients or HDs studied are shown in Figure 3. Nine TCR-BV gene families with biased usage were detected in patients and 10 were detected in HDs. These results show that: (i) BVs 3, 6, 7, 20, 27, and 28 are commonly biased in patients and HDs; (ii) BVs 2, 11, and 15 are biased only in patients; and (iii) BVs 4, 5, 9, and 19 are biased only in HDs.

The usage frequencies of TCR-BV gene families in patients reflect those in HDs. The frequencies of 24 TCR-BV gene families used by T cell receptors (TCRs) of $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells were compared statistically between HLA-A*0201$^+$ patients and HDs (Fig. 4). In all BV gene families, except BVs 5 and 19, the usage frequencies did not differ significantly between patients and HDs, although the subset compositions of $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells were significantly different between the two groups (see Fig. 1c). These results strongly indicate that the frequencies of TCR-BV families used by the TCR of $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells in patients with solid tumors reflect those in HDs.

Discussion

Ratios of $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells with the effector memory phenotype were significantly higher in HLA-A*0201$^+$ patients with solid tumors than in HLA-A*0201$^+$ HDs, while those with the naïve phenotype were significantly lower in patients than in HDs, indicating that $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells were more activated and mature in patients than in HDs. These results are basically compatible with those of our previous study of $WT1_{235}$ $tetramer^+$ $CD8^+$ T cells in HLA-A*2402$^+$ patients with myeloid malignancies and HLA-A*2402$^+$ HDs, where the frequencies of $WT1_{235}$ $tetramer^+$ $CD8^+$ T cells were higher in patients than in HDs and $WT1_{235}$ $tetramer^+$ $CD8^+$ T cells were more activated and mature in patients than in HDs.[30]

In order to analyze TCR-BV gene family usage of the TCRs of human tumor-associated antigen (TAA)-reactive T cells, two methods are routinely used: (i) bulky lymphocyte populations are FACS analyzed using a panel of mAbs directed against individual TCR-BV gene family products; or (ii) the populations are analyzed by PCR using a panel of TCR-BV gene family-specific primers.[34–40] However, the former method does not cover all the BV gene segments distributed in each BV gene family and the latter does not guarantee that all the TCR-BV gene families are amplified from the cDNA with equal efficiency. For example, TCR-BV gene families of T cells that exist at very low frequencies in lymphocytes are easily missed using this sort of PCR method.[40] In contrast, because the present study was performed at the single cell level and because the amplification efficiency of TCR-BV cDNA from a single $WT1_{126}$ $tetramer^+$ $CD8^+$ T cell was >80% (data not shown), our results are thought to directly reflect TCR-BV gene family usage in $WT1_{126}$ $tetramer^+$ $CD8^+$ T cells.

Regardless of a striking difference in WT1-specific CTL responses between patients and HDs, the usage patterns of TCR-BV gene families in patients were similar to those in HDs. That is, patients and HDs shared biased usage of TCR-BV families 3,

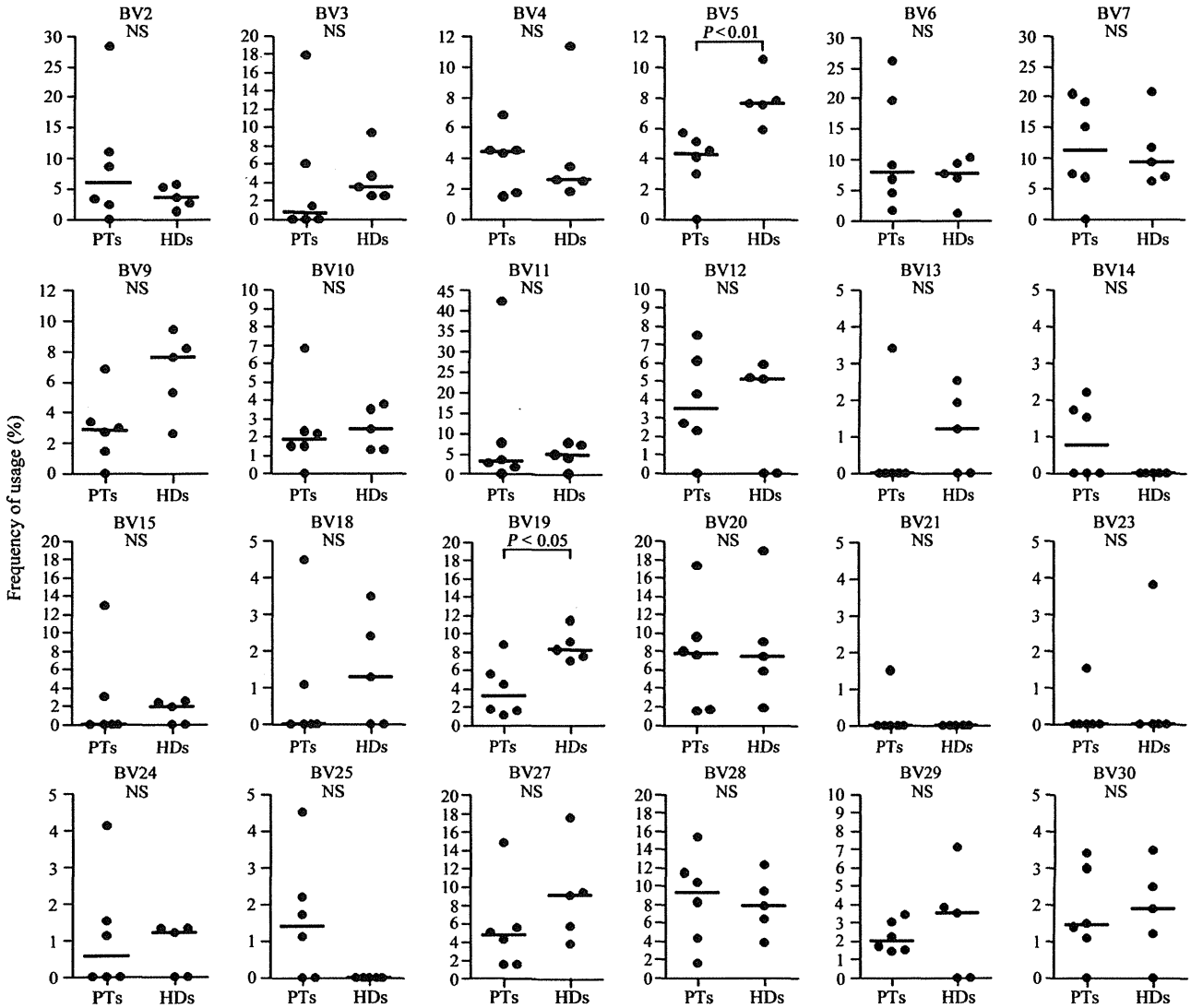

Fig. 4. Statistical comparison of usage frequencies of individual T cell receptor β-chain variable region (TCR-BV) gene families in $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells between patients (PTs) and healthy donors (HDs). The significance of differences was assessed using the Mann–Whitney *U*-test. NS, not significant.

6, 7, 20, 27, and 28, while TCR-BV families 2, 11, and 15 were specifically biased in patients and TCR-BV families 4, 5, 9, and 19 were specifically biased in HDs. In total, six (3, 6, 7, 20, 27, and 28) of ten TCR-BV families (3, 4, 5, 6, 7, 9, 19, 20, 27, and 28) with biased usage in HDs also exhibited biased usage in patients. Three TCR-BV families (2, 11, and 15) newly emerged as those with biased usage specific to patients. However, in all BV gene families, except BVs 5 and 19, the usage frequencies did not differ significantly between patients and HDs. Together, these results led us to speculate that WT1-specific CTLs that had existed predominantly prior to the onset of the solid tumor had expanded and differentiated to maintain their dominance in tumor-bearing patients, whereas a few WT1-specific CTL populations with distinct TCR-BV families expanded in a tumor-bearing patient-specific manner. Furthermore, it may be suggested that WT1-specific CTLs with a dominant set of TCR-BV families in HDs play an important role in immune surveillance against tumors, and that the dominant populations continue to expand due to stimulation of the tumor-derived WT1 protein in WT1-expressing tumor-bearing patients. The immune response to WT1 may be unique, compared with other tumor-associated antigens, in the sense that WT1-specific CTLs are retained in healthy people at relatively higher levels, suggesting that precursors of WT1-specific CTLs are not deleted by the thymus, pass through it, and flow into the periphery. In fact, Pospori *et al.*[41] demonstrated that after murine hematopoietic stem cells transducted with the TCR gene of human HLA-A*0201-restricted WT1-specific CTLs had been transplanted into HLA-A*0201 transgenic recipients, surprisingly WT1-specific CTLs were not impaired by central or peripheral tolerance and, instead, differentiated into memory phenotype T cells. This suggests that precursors of WT1-specific CTLs are not deleted by the thymus. Thus, WT1-specific CTLs are likely to have some role in immune surveillance against tumors in both healthy people and patients with solid tumors. It appears reasonable that TCR-BV families that were appropriately selected for immune surveillance against tumors under healthy conditions were also preferentially used for immune surveillance under tumor conditions.

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The question as to whether different TCR-BV families are used in distinct differentiation subsets of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells was addressed in the present study. To resolve this issue, we analyzed differences in the usage frequencies of individual TCR-BV families between naïve and effector memory phenotypes, which are major and important phenotypes of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. However, only PBMC from HD-3 and -4 were available for this experiment because they were relatively abundant, while those from the other HDs and patients were too few in number to be analyzed. The $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells were divided into four cell populations of naïve, central memory, effector memory, and effector according to the cell surface expression of CCR7 and CD45RA, and both naïve and effector memory cell populations, which included more cells for the analysis, were provided for analysis of TCR-BV families. Eighteen naïve and nine effector memory cells from HD-3 and 26 naïve and 29 effector memory cells from HD-4 were FACS sorted and analyzed. As shown in Figure S1, available as Supplementary Material for this paper, usage frequencies of individual TCR-BV families were analyzed statistically between naïve and effector memory cell populations. In HD-3, no significant differences in usage frequencies of TCR-BV families were observed between naïve and effector memory cell populations. In addition, in HD-4, there were no significant differences in usage frequencies in most (13 of 15) of the TCR-BV families between the two cell populations, although the usage frequencies of only two TCR-BV families (i.e. BVs 12 and 19) were biased ($P = 0.0292$ and $P = 0.0019$, respectively). These results indicate that the usage pattern of TCR-BV families is similar between naïve- and effector memory-typed WT1-specific CTLs. These results also suggest that the patterns of biased usage of TCR-BV families does not change during the differentiation process from naïve to effector through central memory and effector memory.

In both patients and HDs, TCR-BV families 3, 6, 7, 20, 27 and 28 are preferentially used in $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells. As for TCR-BV families of CTLs for other TAAs, it has been reported that, in a melanoma patient, HLA-A2-restricted NY-ESO-1-specific CD8$^+$ T cells preferentially used TCR-BV families 6, 9, and 12.[35] Among these three TCR-BV families, TCR-BV family 6 was also preferentially used by TCRs of $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells in patients and HDs in the present study, while TCR-BV family 9 was preferentially used by $WT1_{126}$ tetramer$^+$ CD8$^+$ T cells in HDs. Thus, it is interesting to observe the phenomenon that a given set of TCR-BV families are preferentially used by certain TAA-specific CD8$^+$ T cells and that some of these families are shared by different TAA-specific CTLs. However, the reason why dominant CTLs for different TAAs (WT1 and NY-ESO-1) shared the same TCR-BV families 6 and 9 is difficult to explain at present. One explanation may be that TAA-specific CTLs with TCR-BV families 6 and 9 have an important role in tumor immunity in the context of HLA-A2 restriction. Further investigations are needed to address this issue.

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Disclosure Statement

The authors have no conflicts of interest.

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