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Advances in Chimeric Antigen Receptor Immunotherapy for Neuroblastoma

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Abstract

Neuroblastoma (NBL) is the most common extracranial pediatric solid tumor and has heterogeneous biology and behavior. Patients with high-risk disease have poor prognosis despite complex multimodal therapy; therefore, novel curative approaches are needed. Immunotherapy is a novel therapeutic approach that harnesses the inherent activity of the immune system to control and eliminate malignant cells. One form of immunotherapy uses chimeric antigen receptors (CAR) to target tumor-associated antigens. CARs are derived from the antigen-binding domain of a monoclonal antibody (MAb) coupled with the intracellular signaling portion of the T cell receptor. CARs can combine the specificity and effectiveness of MAbs with the active bio-distribution, direct cytotoxicity, and long-term persistence of T cells. NBL provides an attractive target for CAR immunotherapy as many of its tumor-associated antigens are not expressed at significant levels on normal tissues, thus decreasing potential treatment related toxicity. Two previous clinical trials utilizing L1-cell adhesion molecule (L1-CAM) and disialoganglioside (GD2) specific CARs (GD2-CAR) have demonstrated safety and anti-tumor efficacy in heavily pretreated relapsed/refractory neuroblastoma patients. Based on these promising results and on improved techniques that can further potentiate CAR therapies, two clinical trials are currently investigating the use of GD2-CARs in children with NBL. Several approaches may further enhance anti-tumor activity and persistence of CAR modified cells, and if these can be safely translated into the clinic, CAR-based immunotherapy could become a viable adjunct or potential alternative to conventional treatment options for patients with NBL.

Introduction

Neuroblastoma (NBL) is the most common extracranial solid tumor in children and the 3rd most common cause of pediatric cancer deaths (Smith *et al*., 2010). NBL is heterogeneous in biology and behavior: spontaneous regression can be seen in neonates with either local or systemic disease, while metastatic disease in patients older than 18 months of age poses a treatment challenge despite the combined use of surgical, radiotherapy, chemotherapy, and

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immunotherapy (Nuchtern *et al*., 2012; Baker *et al*., 2010; Matthay *et al*., 1999; Yu *et al*., 2010). Despite the use of multimodal therapy, there is a significant treatment failure rate, so that novel therapeutic options are necessary.

Although the immune system can recognize and kill malignant cells, cancers often escape this surveillance, spread, and ultimately cause death. Immunotherapy is intended to redirect the immune system to target tumors and tumor-associated antigens, leading to the elimination of malignant cells. Multiple forms of immunotherapy are being used in the clinic, including monoclonal antibodies (MAbs) targeted to tumor cell surface antigens or that disrupt the normal checkpoints that inhibit anti-tumor immune responses, cytokines that modify innate or adaptive immunity, tumor vaccines, and adoptive cellular therapies (Cheung *et al*., 2012; Yu *et al*., 2010; Hank *et al*., 1990; Brahmer *et al*., 2012; Topalian *et al*., 2012; Wolchok *et al*., 2013; Bowman *et al*., 1998; Brenner *et al*., 2000; Pule *et al*., 2008; Louis *et al*., 2011; Park *et al*., 2007). Neuroblastoma provides an attractive target for immunotherapy as it is derived from embryonic neuroectoderm and expresses certain antigens not widely detected in non-embryonic tissues, such as the L1-cell adhesion molecule (L1-CAM), disialoganglioside (GD2), B7H3, and o-acetyl GD2 (Schonmann *et al*., 1986; Cheung, 1991; Zhao and Cheung, 1995; Modak *et al*., 2001; Alvarez-Rueda *et al*., 2011).

Clinically, MAb treatment targeting GD2 antigens improves event-free and overall survival in patients with NBL (Yu *et al*., 2010; Cheung *et al*., 2012), but the anti-tumor effects are limited by passive bio-distribution of the antibody, the half-life of the antibody, and its dependence on other effector mechanisms (i.e., complement dependent lysis or antibody dependent cytotoxicity) to destroy tumor cells. Tumor infiltrating lymphocytes (TILs) are capable of cytotoxic activity in a major histocompatibility complex (MHC) dependent manner, and have been used successfully to treat patients with cancer (Morgan *et al*., 2006; Hunder *et al*., 2008). Unfortunately, many solid tumors, including neuroblastoma, may have abnormal MHC expression and antigen processing function, thus limiting the applicability of this approach (Raffaghello *et al*., 2005; del Campo *et al*., 2012).

Effector lymphocytes can be genetically modified to express chimeric antigen receptors (CARs), which bind to tumor-associated antigens and lead to anti-tumor activity in an MHC-unrestricted manner. CARs are usually generated by joining the heavy and light chain variable regions of a monoclonal antibody with a linker to form a single-chain variable fragment (scFv). The scFv is then attached most commonly to the transmembrane and cytoplasmic portions of the T cell receptor (TCR) ζ chain, via a flexible hinge region, to form a fully functional 1st generation CAR (Figure 1A). Engagement of the scFv results in tyrosine phosphorylation of immunereceptor activation motifs present in the cytoplasmic domain (Savoldo and Dotti, 2013), and nuclear signaling, leading to T cell activation, cytokine secretion, and cellular proliferation. In addition to TCR ζ chain phosphorylation, effective T cell activation requires costimulatory signals from the tumor or professional antigen presenting cells (APCs). The tumor microenvironment lacks fully functional APCs and solid tumors typically do not express costimulatory surface molecules. Therefore, to provide costimulatory signals for optimal activation and survival of adoptively transferred cells, intracellular endodomains from costimulatory receptors such as CD28, OX40, 4-1BB,

or inducible T-cell costimulator (ICOS) have been added to the 1st generation CAR constructs (Figure 1B). The 2nd and 3rd generation CAR products have improved anti-tumor effects, proliferation, and cytokine release in both preclinical and early phase clinical studies (Krause *et al*., 1998; Pule *et al*., 2005; Brentjens *et al*., 2007; Zhong *et al*., 2010; Kalos *et al*., 2011; Porter *et al*., 2011; Savoldo and Dotti, 2013).

Here, we will review the past, present, and future of chimeric antigen receptor based neuroblastoma therapies.

Previous Clinical Trials with Chimeric Antigen Receptors

The first phase 1 clinical trial using CARs for children with NBL investigated the feasibility, safety, and antitumor efficacy of a first generation CAR targeting the cell adhesion molecule L1-CAM in patients with relapsed/refractory disease (Park *et al*., 2007). L1-CAM is a glycoprotein belonging to the immunoglobulin superfamily, located on chromosome Xp28. L1-CAM expression is relatively specific to neuroblastoma, although the molecule is also found on some cells of the central nervous system, sympathetic ganglia, and adrenal medulla, as well as on other types of cancer cells (Euer *et al*., 2005; Arlt *et al*., 2006; Fogel *et al*., 2003; Thies *et al*., 2002; Izumoto *et al*., 1996). The L1-CAM specific 1st generation CAR was constructed using the scFv from the CE7 MAb. Ten heavily pre-treated patients were enrolled in this study. Two patients became ineligible prior to cell infusion, and two cell products did not meet release criteria due to duplicate CAR gene insertion and low level endogenous T cell receptor expression; thus 6 patients received a total of 12 infusions. The characteristics of these patients are summarized in Table 1. Grade III toxicities included lymphopenia, neutropenia, low hemoglobin, bacteremia, and pneumonitis, but there were no Grade IV or V toxicities. Based on the International Neuroblastoma Response Criteria (Brodeur *et al*., 1993), an anti-tumor response was detectable in one patient who had minimal residual disease prior to infusion. The patient first had a mixed and then partial response, 35 and 56 days after cell infusion, respectively, and the infused cells were detectable in the peripheral blood up to 56 days after adoptive transfer. None of the remaining patients had an anti-tumor response and all had shorter persistence of CAR T cells. The authors suggest that the decreased persistence could have been due to an immune response directed toward the fusion protein CE7R although no anti-CE7R serum activity was ever detected (Park *et al*., 2007).

This trial proved the feasibility of expanding T cell clones genetically modified to express a 1st generation CAR for the treatment of children with relapsed/refractory neuroblastoma. One reason for the limited antitumor efficacy seen with this product may have been suboptimal persistence of infused cells associated with inadequate activation and proliferation from the first generation CAR, or exhaustion after relatively long *ex vivo* culturing (Riddell *et al*., 1992).

The largest phase 1 clinical trial for patients with NBL used a GD2-specific 1st generation CAR and evaluated their safety and anti-tumor efficacy (clinicaltrials.gov ID: NCT00085930) (Pule *et al*., 2008; Louis *et al*., 2011). GD2 is a disialoganglioside expressed on tumors such as neuroblastoma, melanoma, and osteosarcoma. GD2 is also expressed on

peripheral nerves and some cells of the cerebellum. The GD2-specific scFv of this CAR was generated from the 14g2a MAb (Rossig *et al*., 2000; 2001). The CAR was inserted into autologous activated T cells (ATCs) or Epstein-Barr virus specific cytotoxic T cells (CAR-CTLs) by retroviral transduction. The investigators hypothesized that persistence of CAR-CTLs may be superior to that of CAR-ATCs as CAR-CTLs would receive additional costimulation from binding at the native EBV-specific TCR. Both ATCs and EBV-CTLs were infused into each patient and peripheral blood was monitored for evidence of persistence and immunophenotypic changes (Pule *et al*., 2008; Louis *et al*., 2011).

On this clinical trial, 19 patients were treated and received a total of 40 products. 8/19 had no evidence of disease at the time of infusion (5/8 after treatment for relapse and 3/8 after treatment for high-risk disease). The infusions were well tolerated. There were no dose limiting toxicities and the only treatment-related adverse events were low-grade fever and mild pain at the sites of known disease. None of the patients developed neurologic abnormalities or peripheral neuropathy (Pule *et al*., 2008; Louis *et al*., 2011). Significant antitumor efficacy was detected including 3 complete remissions, 1 partial response, 1 stable disease, and 2 with tumor necrosis out of the 11 patients with evaluable disease at the time of enrollment. The median overall survival was 931 days and after a median follow-up of 329 days, 2 of the 3 complete response patients were disease free. When comparing CAR-CTLs with CAR-ATCs, the persistence of CAR-CTLs was superior during the first 6 weeks post-infusion; however, longer-term follow-up found that both types of gene modified cell product were at low levels for an extended period of time (up to 96 and 192 weeks, respectively, for CAR-CTL and CAR-ATCs). Importantly, detection of the CAR T cells in peripheral blood for 6 weeks or more was associated with a statistically prolonged time to progression (TTP) (Louis *et al*., 2011), and such detection correlated with the number of CD4 T helper cells and central memory cells in the infused product.

This trial showed that the adoptive transfer of autologous, 1st generation GD2-specific CAR T cells (GD2-CAR) was safe and led to anti-tumor activity in children with high-risk or relapsed/refractory NBL. The results also confirmed that the presence of both central memory cells and CD4 T cell subsets in the T cell product is critical for long-term CAR persistence and that prolonged persistence was associated with improved tumor control.

Current Clinical Investigations with Neuroblastoma Specific CARs

Investigators have evaluated 2 distinct methods to increase persistence: lymphodepletion prior to adoptive transfer and utilization of 2nd or 3rd generation CARs. Lymphodepletion prior to cell infusions creates a better homeostatic environment for the transferred cells to expand (Muranski *et al*., 2006). Newer generation CARs improve both activation and costimulation after antigen binding (Figure 1) (Savoldo and Dotti, 2013). Based on these data, two recently open clinical trials are aiming to improve the effectiveness of CARs for children with NBL.

In one study, 1st generation GD2-CAR donor derived virus-specific CTLs are being used to treat children with relapsed/refractory NBL after allogeneic stem cell transplantation (ASCT) (clinicaltrials.gov ID: NCT01460901). Graft versus tumor (GVT) activity has been

described after ASCT for NBL (Perez-Martinez *et al*., 2009; Matthay *et al*., 1994; Ladenstein *et al*., 2008). Unfortunately, the anti-tumor benefit was neutralized by transplant related mortality (TRM). Viral infections are one of the major cause of mortality after ASCT (Kennedy-Nasser *et al*., 2008) and virus-specific T cells (VSTs) targeting EBV, CMV, and adenovirus have been used successfully to both prevent and treat post-transplant viral infections (Watarai *et al*., 2008; Gerdemann *et al*., 2013; Leen *et al*., 2009). Therefore, investigators hypothesize that using VSTs expressing a 1st generation GD2 CAR may improve the survival of patients with relapsed/refractory NBL by 1) allowing CAR modified cells to take advantage of the lymphodepleted, post-transplant environment to grow and expand after infusion; 2) by providing an antigen-specific GVT effect; 3) decreasing viral-associated TRM by prophylactic adoptive transfer of CAR-expressing tri-virus specific T cells early post-transplant; and 4) by providing additional costimulation to the infused cells via binding at their native T cell receptor upon exposure to viral antigens. This single center phase 1 trial aims to determine the safety profile and the behavior of the infused CAR-VSTs.

The second currently approved study (GRAIN) is a dose escalation trial using ATCs transduced with a 3rd generation GD2-CAR and inducible caspase 9 (iC9) safety switch for children with relapsed/refractory disease (clinicaltrials.gov ID: NCT01822652). The incorporation of CD28 and OX40 costimulatory endodomains into the previously used 1st generation GD2-CAR improved cell survival and antitumor effect during preclinical evaluations (Pule *et al*., 2005). However, other investigators using 2nd and 3rd generation CARs had noted both increased efficacy and increased toxicity (cytokine storm or cytokine release syndrome) once these products were tested clinically (Morgan *et al*., 2010; Kalos *et al*., 2011; Brentjens *et al*., 2013). In order to add an additional layer of safety, the current neuroblastoma CAR-T cell study incorporates an inducible caspase 9 (iC9) safety switch within the construct. iC9 is a genetically modified molecule engineered to dimerize, trigger programmed cell death, and rapidly eliminate the gene modified cell when exposed to the small molecule AP1903. Activation of the iC9 construct after AP1903 infusion has been tested in a previous immunotherapeutic clinical trial for pediatric patients and led to the elimination of 90% of transduced cells within 30 minutes of the IV infusion (Di *et al*., 2011).

Future Directions

Future efforts will likely focus on increasing the activity of the infused CAR-T cells, improving their targeting, and reducing their sensitivity to the inhibitory microenvironment of the tumor.

To broaden the utility of CAR immunotherapy in the solid tumor setting, many research teams are looking for ways to further enhance anti-tumor efficacy while maintaining an acceptable toxicity profile. For example, alternative or additional costimulatory endodomains such as 4-1BB or ICOS may provide a CAR construct that provides superior survival and activation signals for anti-tumor activity in the immunosuppressive microenvironment fostered by NBL.

Further, as the affinity of CARs and/or TCRs increases with genetic modification, the likelihood of on- and off-target toxicity secondary to low level antigenic expression on normal tissues increases. Thus, further discovery and evaluation of tumor specific antigens, as well as improved methods of preclinical toxicity assessments will be critically important (Linette *et al*., 2013). L1-CAM and GD2 expression is not strictly restricted to NBL; however, the recently described o-acetyl-GD2 (oaGD2) has been found on 100% of NBL cells and its expression appears completely absent on peripheral nerves (Alvarez-Rueda *et al*., 2011). Using oaGD2 as a CAR target may maintain anti-tumor effects without increasing the risk of neuronal toxicity and preclinical testing is on-going. Improving tumor trafficking by adoptively transferred CAR cells may also increase their effectiveness (Di *et al*., 2011). For example, NBL produces CCL2 and genetic modification of GD2-CAR T cells with the receptor for this chemokine (CCR2b) improved T cell homing to the tumor and resulted in better *in vivo* and *in vitro* antitumor activity (Craddock *et al*., 2010).

A complex interplay exists between malignant NBL cells and non-malignant stromal cells that leads to an overall immunosuppressive microenvironment (Pistoia *et al*., 2013). Preclinical testing has shown that this inhibitory environment can be overcome by stronger activation of T cell intracellular survival pathways (i.e., constitutive activation of akt), the production of cytokines locally by CAR modified cells (i.e., designed to secrete IL-7 or IL-15), or genetic engineering of the cells with non-functional TGF-β receptors (Sun *et al*., 2010; Hoyos *et al*., 2010; Perna *et al*., 2013; Foster *et al*., 2008). Conversely, constitutive pathway activation or autocrine cytokine secretion may allow gene-modified cells to evade physiologic control of proliferation and survival. The use of a suicide gene or the development of transient expression of these modifications may be important alternatives to limit the risk of uncontrolled cell proliferation and maintain safety.

Lastly, investigators are also looking at ways to optimize the cellular product that is given to patients. For example, the presence of CD4 T helper cells and/or central memory T cells (CM) within the infused product has been associated with improved persistence and antitumor activity of CAR T cells in both preclinical and clinical studies (Louis *et al*., 2011; Klebanoff *et al*., 2012). With recent advances in GMP manufacturing, clinical scale expansion of either high purity CD4 T cells or CM T cells is possible and genetic engineering of these cells with an NBL-specific CAR may provide an effective treatment approach. Additionally, other cell types besides T cells can be genetically modified to express CARs. Natural killer T cells (NKTs) are an evolutionary conserved subset of innate lymphocyte expressing an invariant TCR (Valfa24, Jalfa18, paired with Vbeta11 in humans). They react with monomorphic CD1d on antigen presenting cells such as monocytes/ macrophages (Kronenberg and Gapin, 2002). NKTs control NBL growth by destroying cancer supporting tumor associated macrophages (TAMs) in the tumor microenvironment (Song *et al*., 2009) and the presence of NKTs in primary NBL tissues from patients with Stage 4 disease is associated with improved survival. NKTs genetically modified to express GD2-CARs are showing promising results in preclinical models by killing both NBL and TAMs *in vitro* and inducing significant anti-tumor effect *in vivo* (Heczey *et al*., 2013). Natural killer (NK) cells have innate antitumor properties and killer inhibitory receptor mismatched NK cells were shown to have anti-tumor effect post-

autologous transplantation in children with NBL (Perez-Martinez *et al*., 2009). Genetic modification of NK cells with either a 14g2a scFv and the NK cell activating 2B4 intracellular domain, or 14.18 scFv and TCR ζ chain, have both shown promising preclinical activity (Altvater *et al*., 2009; Esser *et al*., 2012).

Conclusion

Previous studies using CARs to treat patients with NBL have shown safety and promising anti-tumor effects. A number of approaches have been developed to further enhance anti-tumor activity of CAR modified cells. If these enhancements can be safely translated into the clinic, using CARs to treat NBL can become a viable non-chemotherapeutic treatment alternative for patients diagnosed with this disease.

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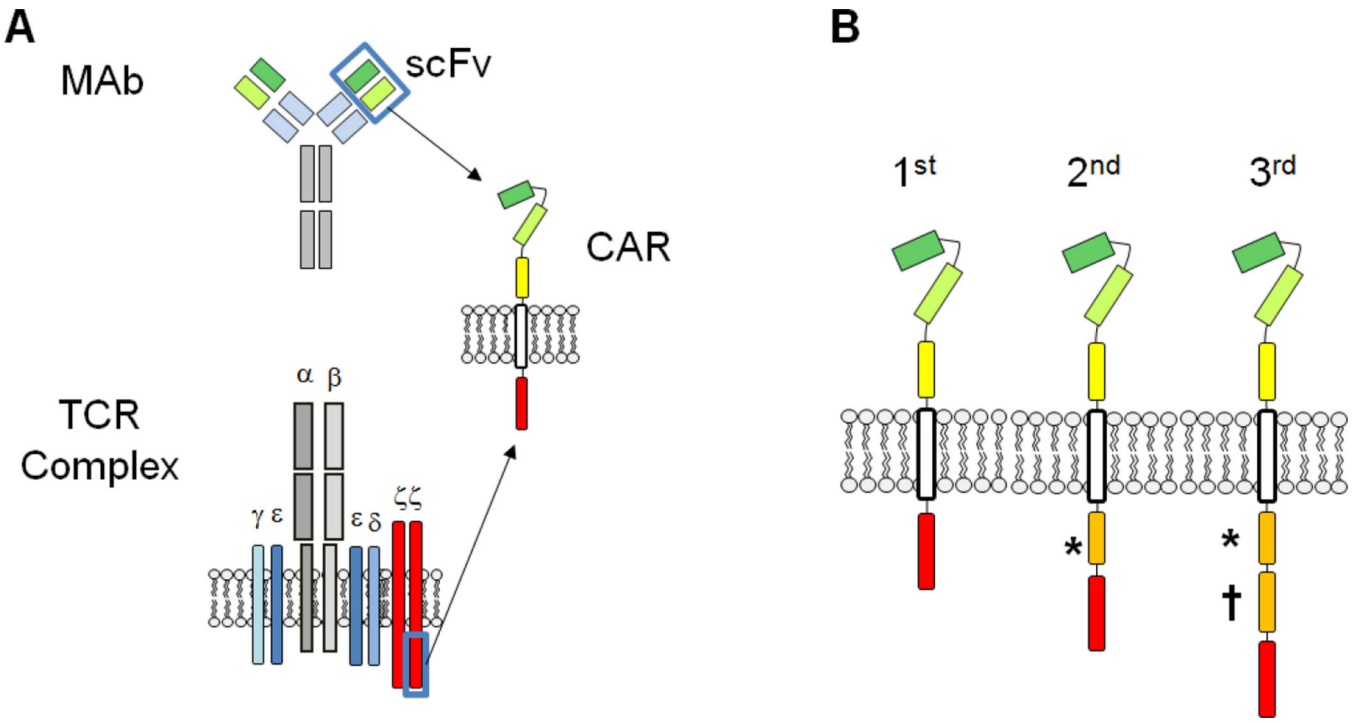

Figure 1.

Structure and design of chimeric antigen receptors (CARs). A) The extracellular, antigen binding domain of a CAR is typically derived from the single chain variable fragment (scFv) of a monoclonal antibody (MAb). The scFv segment is further linked to a transmembrane domain and an endodomain -- most commonly the signaling moiety of the T cell receptor (TCR) zeta chain. B) CARs are classified into 1st, 2nd, or 3rd generation products depending on the number of costimulatory endodomains (*, †) added to the construct. The most commonly used costimulatory endodomains in clinical and preclinical studies are CD27*, CD28*, inducible T-cell costimulator (ICOS)*, 4-1BB†, and OX40†.

Table 1

Patient Characteristics.

Study	Patient #	Age	Gender	Disease Status	Cell Dose	Response at Day 56	Overall Response	Outcome[1]
L1-CAM CAR T cell trial[2]	1	9	M	Relapsed, bone, bone marrow	10^8, 10^9	PD	CR	DOD
	2	12	M	Relapsed, bulky	10^8, 10^9	PD	PD	DOD
	3	7	F	Relapsed, bone	10^8, 10^8	PR	PD	DOD
	4	9	F	Relapsed, bone, LN	10^8	PD	PD	DOD
	5	16	F	Relapsed, bulky	10^8, 10^9	PD	SD	DOD
	6	10	M	Relapsed, bulky	10^8, 10^8, 10^8	PD	PD	DOD
GD2-CAR EBV-CTL/ATC trial[3]	1	9	M	NED	$2\times10^7/m^2$	NED	NED	NED
	2	5	M	NED	$2\times10^7/m^2$	NED	NED	NED
	3	4	M	NED	$2\times10^7/m^2$	NED	NED	NED
	4	20	F	Relapsed, NED	$2\times10^7/m^2$	NED	NED	AWD
	5	7	M	Relapsed, NED	$2\times10^7/m^2$	NED	NED	AWD
	6	4	F	Relapsed, bone	$2\times10^7/m^2$	PD	PD	DOD
	7	9	F	Relapsed, bone	$2\times10^7/m^2$	CR	CR	CR
	8	4	F	Relapsed, bone	$2\times10^7/m^2$	PR	CR	CR
	9	10	M	Relapsed, bulky	$2\times10^7/m^2$	PD	PD	DOD
	10	11	M	Relapsed, bulky	$2\times10^7/m^2$	PD	PD	DOD
	11	10	F	Relapsed, NED	$5\times10^7/m^2$	NED	NED	NED
	12	4	F	Relapsed, NED	$5\times10^7/m^2$	NED	NED	DOD
	13	15	F	Relapsed, bone marrow	$5\times10^7/m^2$	CR	CR	DOD
	14	9	F	Relapsed, bulky	$5\times10^7/m^2$	PD	PD	DOD
	15	3	M	Relapsed, bulky	$5\times10^7/m^2$	SD	SD	DOD
	16	9	F	Relapsed, bulky	$5\times10^7/m^2$	Tumor necrosis	Tumor necrosis	DOD
	17	7	M	Relapsed, NED	$1\times10^8/m^2$	NED	NED	DOD
	18	4	F	Relapsed, bulky	$1\times10^8/m^2$	Tumor necrosis	Tumor necrosis	DOD
	19	7	M	Relapsed, bulky	$1\times10^8/m^2$	SD	PR	AWD

Abbreviations: M, male; F, female; NED, no evidence of disease; Bulky, residual disease not only bone, bone marrow, or lymph node; LN, lymph node; AWD, alive with disease; DOD, died of disease; PD, progressive disease; SD, stable disease; PR: partial response; CR: complete response; Overall response: Response after salvage / best response;

Note:

[1], at the time of publications;

[2], Park *et al*., 2007;

[3], Pule *et al*., 2008 and Louis *et al*., 2011.