BioMed Research International

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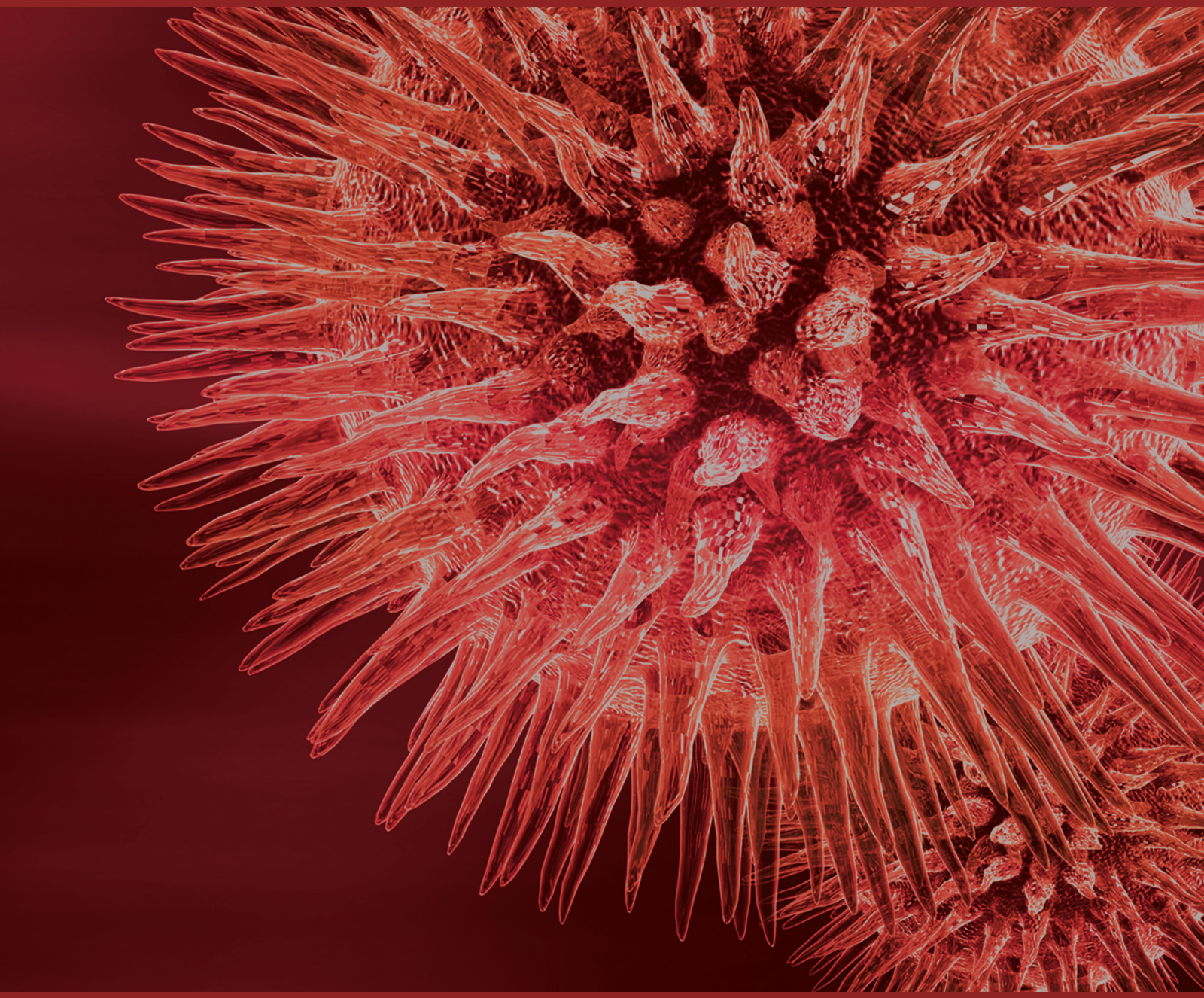
Guest Editors: Haiqing Ma, Saradhi Mallampati, Gang An, and Jin Wang



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Editorial

Targeted Therapy in Hematological Malignancies: From Basic Research to Clinical Practice

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Targeted therapy in hematological malignancies has been a forerunner and still remains in the forefront of ongoing research. Over the past decades, many advances in basic research have boosted the advancement of targeted therapy in clinical studies. To date, targeted therapy has provided benefits for patients with hematological malignancies either as the first-line treatment or in combination with chemotherapy. Undoubtedly, in the near future, customized targeted therapy will play a more important role in the treatment of hematological malignancies.

This special issue on targeted therapy in hematological malignancies includes reviews and original research articles that describe novel molecular targets, innovative technologies, recent clinical trials, mechanisms of drug resistance, and other advances in targeted therapy for hematological malignancies.

Dr. J.-F. Rossi in the review article entitled "Targeted Therapies in Adult B-Cell Malignancies" summarizes currently targeted molecules in adult B-cell malignancies and didactically describes the various cell compartments (membrane versus cytosol) that can be targeted and explains how most of the molecular pathways either proximal or distal to B-cell receptor (BCR) can be blocked with targeted therapies. The review also includes a highly informative synopsis of all the relevant clinical trials and will be extremely useful to all the readers, especially to those in the field of hematology oncology, both in the clinic and in research.

Dr. O. Annibali et al. report the outcome of using Rituximab as a first-line systemic treatment in a series of mucosa-associated lymphoid tissue-type ocular adnexal lymphomas (MALT OALs) with additional maintenance. OALs are rare types of lymphoma, for which the specific treatment options were not currently available. Only few cases were reported previously on the efficacy of Rituximab immunotherapy as a single-agent in primary localized MALT OALs. The response duration in the previously reported trials was short which could have been due to the absence of additional maintenance in those studies. This study clearly indicates that the maintenance therapy with Rituximab ensures prolonged remission.

Dr. D. Tusé et al. report the evaluation of novel plant-based conjugate vaccines for targeted treatment of B-cell follicular lymphoma (FL) in a phase I safety and immunogenicity clinical study. This phase I study was exceedingly successful as none of the patients suffered any serious adverse events related to vaccination. The customized idiotype vaccines produced by means of the magnICON, a plant-based expression technology, are very promising for they are readily and economically manufactured, safe, well tolerated, and immunogenic.

Dr. Z.-X. Yan et al. report that overexpression of miR181 in human T-cell leukemia/lymphoma is related to increased AKT phosphorylation. Malignant T cells overexpressing miR181 exhibited multiple chemoresistance mechanisms through modulation of AKT activity. Moreover, in isogenic

doxorubicin-resistant cell lines developed, the relative resistance to doxorubicin and other chemotherapeutic agents was associated with increased miR181 expression and subsequent AKT activation. So miR181 could serve as a useful biomarker and a potential therapeutic target in treating T-cell malignancies resistant to chemotherapy.

Dr. S. Wu et al. demonstrate that subcutaneous administration of bortezomib is not inferior to its intravenous administration and confirmed that bortezomib and thalidomide plus dexamethasone regimen is highly active and well tolerated as induction therapy in patients with multiple myeloma.

With all these novel and well-done research and clinical trials, this issue promises to be an enlightening read for clinicians and scientists.

Acknowledgment

We would like to thank all authors who submitted their work for this special issue.

Haiqing Ma
Saradhi Mallampati
Gang An
Jin Wang

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Research Article

MicroRNA181a Is Overexpressed in T-Cell Leukemia/Lymphoma and Related to Chemoresistance

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MicroRNAs (miRs) play an important role in tumorogenesis and chemoresistance in lymphoid malignancies. Comparing with reactive hyperplasia, miR181a was overexpressed in 130 patients with T-cell leukemia/lymphoma, including acute T-cell lymphoblastic leukemia ($n = 32$), T-cell lymphoblastic lymphoma ($n = 16$), peripheral T-cell lymphoma, not otherwise specified ($n = 45$), anaplastic large cell lymphoma ($n = 15$), and angioimmunoblastic T-cell lymphoma ($n = 22$). Irrespective to histological subtypes, miR181a overexpression was associated with increased AKT phosphorylation. *In vitro*, ectopic expression of miR181a in HEK-293T cells significantly enhanced cell proliferation, activated AKT, and conferred cell resistance to doxorubicin. Meanwhile, miR181a expression was upregulated in Jurkat cells, along with AKT activation, during exposure to chemotherapeutic agents regularly applied to T-cell leukemia/lymphoma treatment, such as doxorubicin, cyclophosphamide, cytarabine, and cisplatin. Isogenic doxorubicin-resistant Jurkat and H9 cells were subsequently developed, which also presented with miR181a overexpression and cross-resistance to cyclophosphamide and cisplatin. Meanwhile, specific inhibition of miR181a enhanced Jurkat and H9 cell sensitivity to chemotherapeutic agents, further indicating that miR181a was involved in acquired chemoresistance. Collectively, miR181a functioned as a biomarker of T-cell leukemia/lymphoma through modulation of AKT pathway. Related to tumor cell chemoresistance, miR181a could be a potential therapeutic target in treating T-cell malignancies.

1. Introduction

Malignancies derived from the T-cell lineages encompass a heterogeneous group of neoplasm. The World Health Organization (WHO) classification recognizes distinctive subtypes of immature T-cell malignancies, like acute T-cell lymphoblastic leukemia (T-ALL) and T-cell lymphoblastic lymphoma (T-LBL), as well as mature T-cell malignancies, mainly including peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS), anaplastic large cell lymphoma (ALCL), and angioimmunoblastic T-cell lymphoma (AITL) [1]. Varied from clinicopathological features and biological behavior, they are generally more aggressive than their B-cell counterpart, characterized by resistance to conventional chemotherapy and poor prognosis of the patients [2]. Therefore, biomarkers related to tumor progression and chemoresistance remain to be investigated and may become potential targets for future therapy in T-cell leukemia/lymphoma.

MicroRNAs (miRs), a class of 19- to 23-nucleotide noncoding RNA molecules, regulate gene expression by targeting mRNA at the 3′ untranslated region (UTR) [3]. Growing evidences suggested that miRs are critical regulators in tumorigenesis and drug resistance [4, 5]. MiR181 is essential for lymphocyte differentiation and maturation in thymus [6].

More recently, it has been reported that miR181 overexpression promotes cell proliferation and activates PI3K/AKT signaling transduction pathway [7, 8]. Activated in lymphoid malignancies [9], AKT plays a pivotal role in tumor progression and resistance to chemotherapeutic agents [10, 11]. Here we assessed miR181a expression, as well as its relation to AKT activation and chemoresistance in T-cell leukemia/lymphoma.

2. Patients and Methods

2.1. Patients. One hundred and thirty patients diagnosed with T-ALL or T-cell lymphoma were enrolled in this study, including 32 T-ALL, 16 T-LBL, 45 PTCL-NOS, 15 ALCL, and 22 AITL. Histologic diagnoses were established according to the WHO classification [1]. PTCL cases (PTCL-NOS, ALCL, and AITL) were treated with CHOP-based chemotherapy. T-LBL and T-ALL cases were treated with HyperCVAD-A/B regimens as previously reported [12, 13]. Response rates were assessed according to the criteria as reported [12, 13]. The clinicopathological data of the patients was listed in Table 1. Thirty-four age- and sex-matched cases with reactive hyperplasia were referred to as controls. The study was approved by the Institutional Review Board with informed consent obtained in accordance with the Declaration of Helsinki.

2.2. Cell Lines and Reagents. T-leukemia/lymphoma cell lines Jurkat, H9, and embryonic kidney cell line HEK-293T were available from American Type Culture Collection (Manassas, VA, USA). Doxorubicin-resistant Jurkat and H9 cells were established by exposure to gradually increasing concentrations of doxorubicin *in vitro*, as described by Huang et al. [14].

2.3. Cell Proliferation Assay. Cell proliferation was measured by MTT and EdU incorporation assay. Cells were seeded in 96-well plates and incubated with the indicated concentrations of reagents at 37°C. After 72 h incubation, 0.1 mg of MTT was added to each well and the absorbance was measured at 490 nm by spectrophotometry. EdU assay was conducted using Cell-Light EdU imaging kit (RiboBio, Guangzhou, China) according to the manufacturer's instruction.

2.4. MiR181a Detection. Total RNA was extracted from 20 μm thick paraffin ($n = 100$) or frozen sections ($n = 64$) using RecoverAll total nucleic acid isolation kit or Trizol agent following the manufacturer's protocol. MiR181a expression was analyzed by real-time quantitative RT-PCR using miRNA reverse transcription kit, hsa-miR 181a assay, and 7500HT Fast Real-time PCR system (Applied Biosystems, CA, USA). RNU24 was used as endogenous control and Jurkat cells for calibration. A relative quantification was calculated using the $^{\Delta\Delta}$CT method [15].

2.5. Western Blot. Cells were lysed in 200 μL lysis buffer (0.5 M Tris-HCl, pH 6.8, 2 mM EDTA, 10% glycerol, 2% SDS, and 5% β-mercaptoethanol). Protein extracts (20 μg) were electrophoresed on 10% SDS polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dried milk in Tris-buffered saline and incubated for 2 h at room temperature with appropriate primary antibody, followed by horseradish peroxidase-conjugated secondary antibody. The immunocomplexes were visualized using chemiluminescence phototope-horseradish-peroxidase kit. Actin was used to ensure equivalent protein loading. Antibodies against phosphorylated-AKT (p-AKT), AKT, actin, and chemiluminescence phototope-horseradish-peroxidase kit were obtained from Cell Signaling (Beverly, MA, USA). Anti-PTEN antibody was from Abcam (Cambridge, UK). Horseradish peroxidase-conjugated goat anti-mouse-IgG and goat anti-rabbit-IgG antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.6. Cell Transfection. HEK-293T cells were incubated with pEZX-181a vector (HmiR0292-MR03) or a control vector pEZX-ct (CmiR0001-MR03, Genecopia, MD, USA) and Li pofectamine 2000 (Invitrogen, CA, USA) for 24 h and replaced in fresh medium for further experiments. To inhibit miR181a expression, Jurkat and H9 cells were transfected with 10 nM antagomir using Lipofectamine 2000 (Invitrogen) for

Table 1: Clinicopathological characteristics of 130 patients with T-cell leukemia/lymphoma.

Characteristics	High miR181a expression ($n = 65$)	Low miR181a expression ($n = 65$)	P value
Age (years)			
≥60	13	17	0.5328
<60	52	48	
Gender			
Male	42	32	0.1106
Female	23	33	
Lactic dehydrogenase level (LDH)			
Normal	19	23	0.5740
Above normal	46	42	
Pathological subtypes			
T-ALL	15	17	
T-LBL	8	8	
PTCL-NOS	22	23	0.6656
ALCL	6	9	
AITL	14	8	
International prognostic index (IPI)			
Low	12	11	
Low/intermediate	23	33	0.3152
Intermediate/high	20	15	
High	10	6	
Overall response (CR + PR)	38	50	0.0385

*CR, complete remission; PR, partial remission.

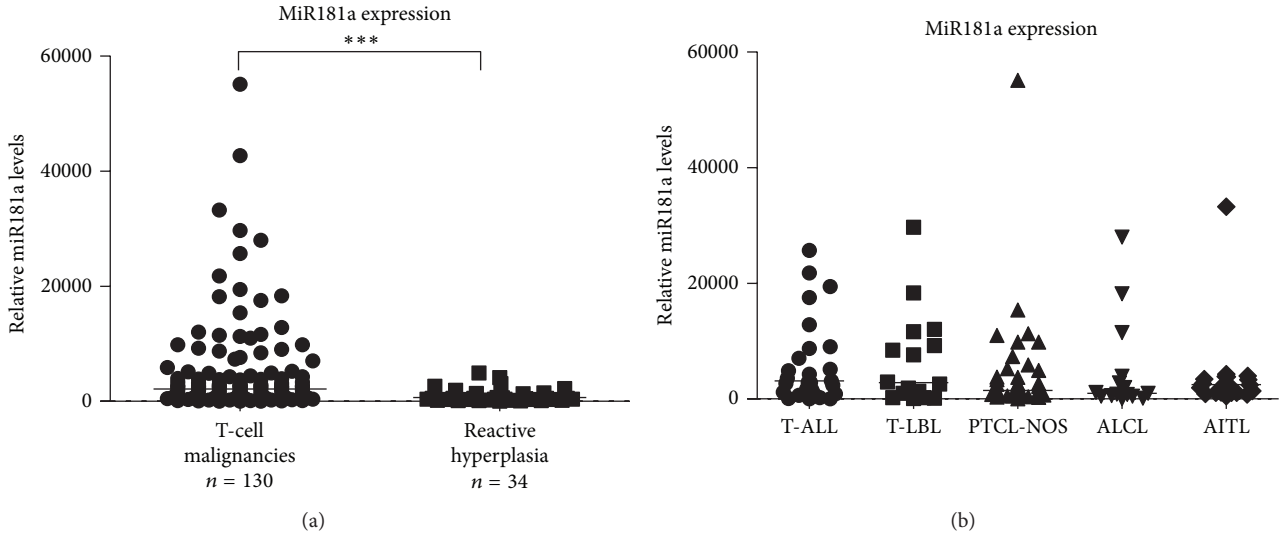

Figure 1: MiR181a was overexpressed in T-cell leukemia/lymphoma. (a) As detected by real-time quantitative PCR, miR181a was overexpressed in T-cell malignancies. $^{***}P < 0.001$ comparing with reactive hyperplasia. The relative expression level of each patient was calculated based on the lowest expression value. (b) MiR181a was overexpressed in acute T-cell lymphoblastic leukemia (T-ALL, $n = 32$), as well as subtypes of T-cell lymphoma, including T-cell lymphoblastic lymphoma (T-LBL, $n = 16$), peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS, $n = 45$), anaplastic large cell lymphoma (ALCL, $n = 15$), and angioimmunoblastic T-cell lymphoma (AITL, $n = 22$).

24 h. The miR181a antagomir and the negative control were synthesized by Shanghai Biotend Biotechnologies Co., Ltd. (Shanghai, China).

2.7. Immunohistochemistry Assay. Immunohistochemistry was performed on 5 μm paraffin sections with an indirect immunoperoxidase method using antibodies against p-AKT (Cell Signaling). Expression levels were scored semiquantitatively based on percentage of positive cells: +, <25%; ++, 25–49%; +++, 50–74%; ++++, 75–100%.

2.8. Statistical Analysis. Differences of miR181a expression among groups were assessed using Mann-Whitney U test. The association between miR181a and p-AKT expression in human tumor samples was analyzed by Fisher's exact test. *In vitro* experimental results were expressed as mean ± S.D. of data obtained from three separate experiments and determined using t-test to compare variance. $P < 0.05$ was considered statistically significant.

3. Results

3.1. MiR181a Was Overexpressed in T-Cell Leukemia/Lymphoma and Related to AKT Activation. Compared with reactive hyperplasia, miR181a was overexpressed in T-cell leukemia/lymphoma ($P < 0.0001$, Figure 1(a)). No significant difference was observed among T-ALL and subtypes of T-cell lymphoma ($P = 0.5153$, Figure 1(b)).

The median value of relative miR181a expression in T-cell leukemia/lymphoma was 2136. The patients with miR181a expression level over and equal to the median value were regarded as high miR181a expression, whereas those below the median value were included in the low miR181a expression. Patients with high miR181a expression had significantly lower overall response rate (ORR) than those with low miR181a expression (Table 1). P-AKT expression was detected by immunohistochemistry in primary tumor sections of 12 T-cell lymphoma patients (6 cases from high miR181a expression group and 6 cases from low miR181a expression group, Figure 2(a)). High miR181a expression was associated with increased positivity of p-AKT ($P = 0.0152$, Figure 2(b)).

3.2. MiR181a Promoted Cell Proliferation and Induced Chemoresistance through Activating AKT. T-leukemia/lymphoma cell lines Jurkat and H9 possessed higher levels of miR181a expression than that of HEK-293T cells ($P = 0.0023$ and $P = 0.0030$, resp., Figure 3(a)). To gain insight into the biological function of miR181a, HEK-293T cells, with lowest miR181a expression, were transiently transfected with miR181a (pEZX-181a, Figure 3(b)). Ectopic expression of miR181a remarkably accelerated cell growth, as compared to the control cells (pEZX-ct). In parallel with increased cell proliferation, the percentage of EdU-positive cells was significantly higher in pEZX-181a cells (52.7%±8.7%) than in pEZX-ct cells (20.7%± 7.0%, $P = 0.0458$, Figure 3(c)). Of note, overexpression of miR181a increased AKT phosphorylation, while the total protein level remained constant (Figure 3(d)). AKT is the key regulator of cell proliferation and drug resistance [11, 16]. Accordingly, the IC50 of doxorubicin was significantly increased in the miR181a-overexpressing HEK-293T cells, as compared to the control cells (21.3 ± 3.1 nM versus 9.8 ± 2.3 nM, $P = 0.0260$, Figure 3(e)).

3.3. MiR181a Overexpression Corresponded to Chemoresistance in T-Leukemia/Lymphoma Cells. Doxorubicin (DOX),

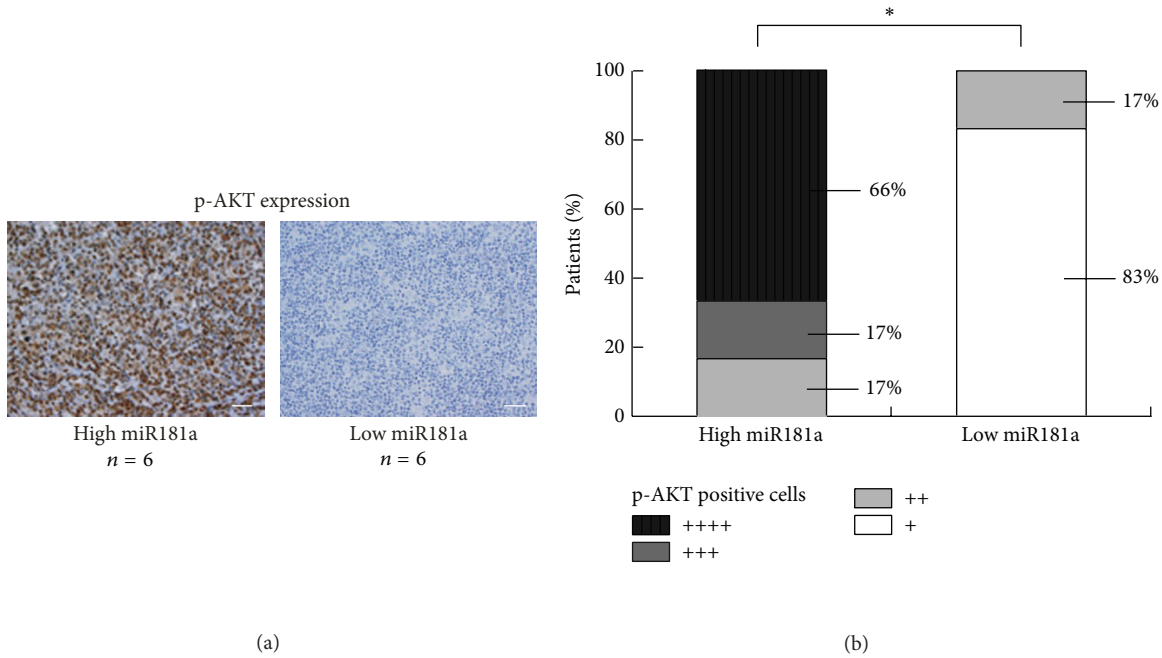

Figure 2: MiR181a overexpression was related to AKT activation in T-cell leukemia/lymphoma. As revealed by immunohistochemistry (a), increased positivity of p-AKT was observed in primary tumor samples of T-cell lymphoma patients with high miR181a expression ($n = 6$), compared to those with low miR181a expression ($n = 6$) (b). $^*P < 0.05$ comparing with low miR181a expression. Bar = 50 μm.

cyclophosphamide (CTX), cytarabine (Ara-C), and cisplatin are main chemotherapeutic agents used in treating T-cell malignancies. When Jurkat cells were exposed to these agents for 48 h, miR181a expression was significantly increased ($P = 0.0019$, $P = 0.0016$, $P = 0.0172$, and $P < 0.0001$, resp., Figure 4(a)), in accordance with increased AKT phosphorylation detected by Western blot (Figure 4(b)).

Acquired drug resistance is an important obstacle that impairs the success of cancer treatment. Isogenic doxorubicin-resistant sublines were developed as previously reported [14, 17] at the concentrations of 7.5 nM (Jurkat/7.5 nM DOX) and 15 nM (Jurkat/15 nM DOX) in Jurkat cells, as well as 5 nM (H9/5 nM DOX) and 10 nM (H9/10 nM DOX) in H9 cells. Compared with the parental cells (Jurkat cells, 44.3 ± 4.0 nM; H9 cells, 14.7±5.5 nM), IC50 of doxorubicin was significantly increased in Jurkat/7.5 nM DOX and Jurkat/15 nM DOX cells (70.1±8.0 nM and 100.0±8.0 nM, $P = 0.0453$ and $P = 0.0152$, resp., Figure 4(c)) and in H9/5 nM DOX and H9/10 nM DOX cells (28.0 ± 4.3 nM and 41.3 ± 4.7 nM, $P = 0.0481$ and $P = 0.0443$, resp., Figure 4(d)). Accordingly, levels of miR181a expression were significantly higher in doxorubicin-resistant cells than in the parental cells (1.8 ± 0.2-fold in Jurkat/7.5 nM DOX and 2.8±0.3-fold in Jurkat/15 nM DOX cells, $P = 0.0253$ and $P = 0.0112$, Figure 4(e); 2.1 ± 0.6-fold in H9/5 nM and 3.4 ± 0.2-fold in H9/10 nM DOX cells, $P = 0.0384$ and $P = 0.0032$, resp., Figure 4(f)), consistent with AKT activation (Figures 4(g) and 4(h)). Increased miR181a expression was linked to AKT phosphorylation, not only in PTEN-negative Jurkat cells [18] but also in PTEN-positive H9 cells (Figures 4(g) and 4(h)). Therefore, regulation of AKT phosphorylation could be independent of PTEN expression in T-cell leukemia/lymphoma. Further drug-sensitivity test showed that these resistant sublines with miR181a overexpression also had cross-resistance to other chemotherapeutic agents (Table 2). Therefore, exposure to chemotherapeutic agents could induce miR181a expression and AKT activation, which is closely related to acquired chemoresistance.

3.4. MiR181a Inhibition Enhanced T-Leukemia/Lymphoma Cell Sensitivity to Chemotherapeutic Agents. Specific inhibition of miR181a in Jurkat and H9 cells as well as their resistant sublines, using an antagomir, significantly increased cell sensitivity to doxorubicin (Figures 5(a) and 5(b)) and decreased AKT phosphorylation (Figures 5(c) and 5(d)). Similar to doxorubicin, decreased miR181a expression was also related to reduced IC50 of cisplatin and cyclophosphamide in doxorubicin-resistant Jurkat and H9 cells (Table 3).

4. Discussion

In addition to genetic abnormalities, epigenetic aberrations, particularly those of miRs, participate in human carcinogenesis [5, 19]. MiR181a is critically involved in hematological malignancies. In acute myelogenous leukemia, increased miR181a expression was significantly associated with a higher complete remission rate of the patients [20]. However, as recently reported in lymphoid malignancies like acute lymphoblastic leukemia and multiple myeloma, miR181 overexpression was related to advanced stage and tumor progression [7, 21]. In accordance with malignant T cells, miR181a is

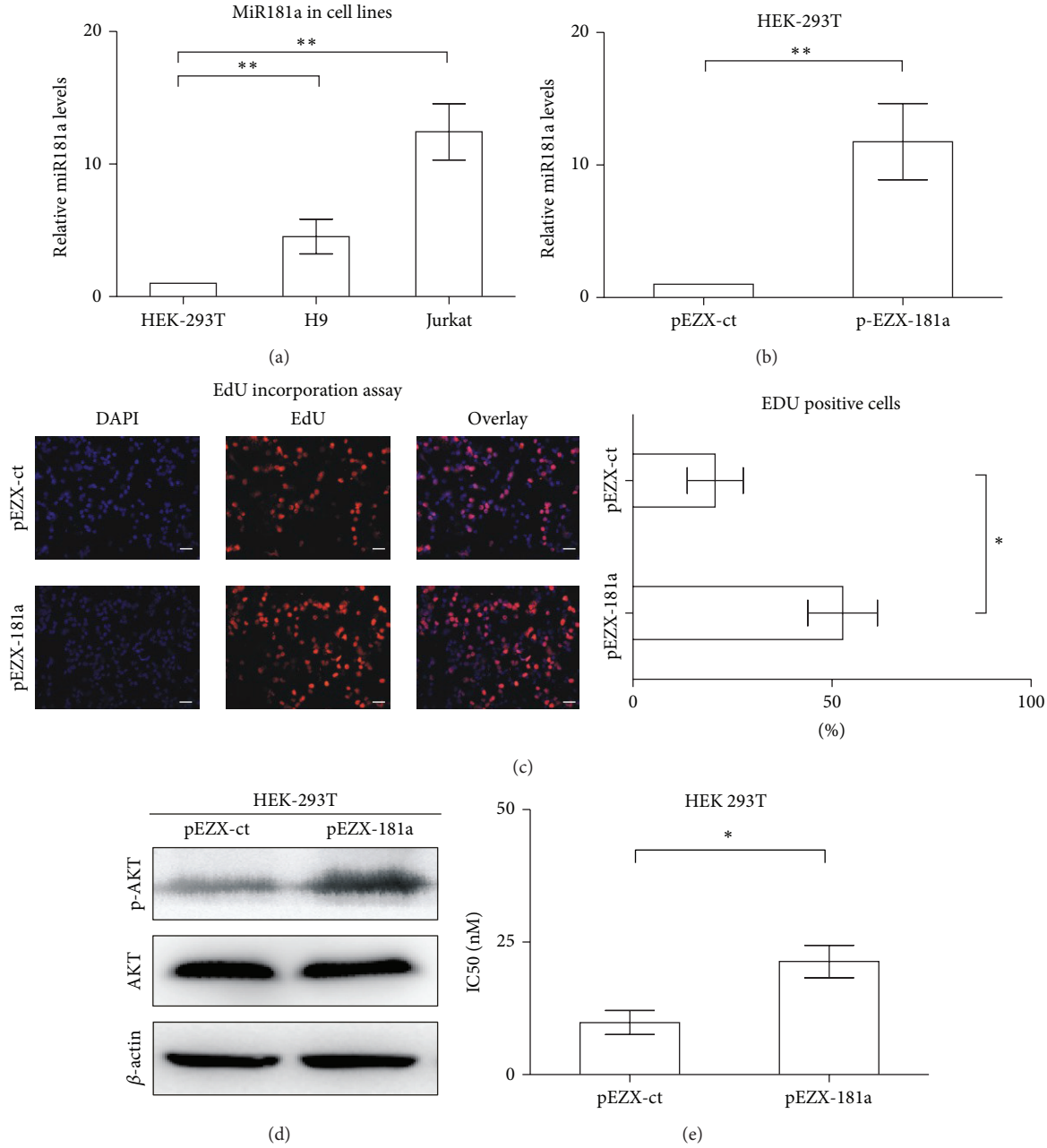

FIGURE 3: Ectopic expression of miR181a enhanced cell proliferation and resistance to doxorubicin through AKT activation. (a) T-leukemia/lymphoma Jurkat and H9 cells had significantly higher expression levels of miR181a than that of HEK-293T cells. $^{**}P < 0.01$ comparing with HEK-293T cells. (b) Transfection with miR181a (pEZX-181a) in HEK-293T cells resulted in significantly increased miR181a expression. $^{**}P < 0.01$ comparing with the control pEZX-ct cells. (c) EdU incorporation assay in HEK-293T cells showed that miR181a-overexpressing pEZX-181a cells presented with increased EdU-positive cells. $^{*}P < 0.05$, comparing with the control pEZX-ct cells. Bar = 20 μm. (d) Overexpression of miR181a increased AKT phosphorylation, while the total protein level remained constant. (e) IC50 of doxorubicin was significantly higher in the pEZX-181a cells than in the control pEZX-ct cells. $^{*}P < 0.05$ comparing with the control pEZX-ct cells.

upregulated in normal T-cell counterpart and miR181a/b-deficient mice show severe defects in T-cell development [6, 8]. Therefore, miR181 may have different roles in hematological malignancies. Our study showed that miR181a, independent of histological subtypes, was overexpressed and these patients were less responding to treatment, referring to miR181a as a common biomarker of chemoresistance in T-cell leukemia/lymphoma.

Chemoresistance, determining therapeutic effect and clinical outcome of the patients, is one of the control factors in cancer treatment, including T-cell leukemia/lymphoma [22]. Here miR181a was closely related to chemoresistance

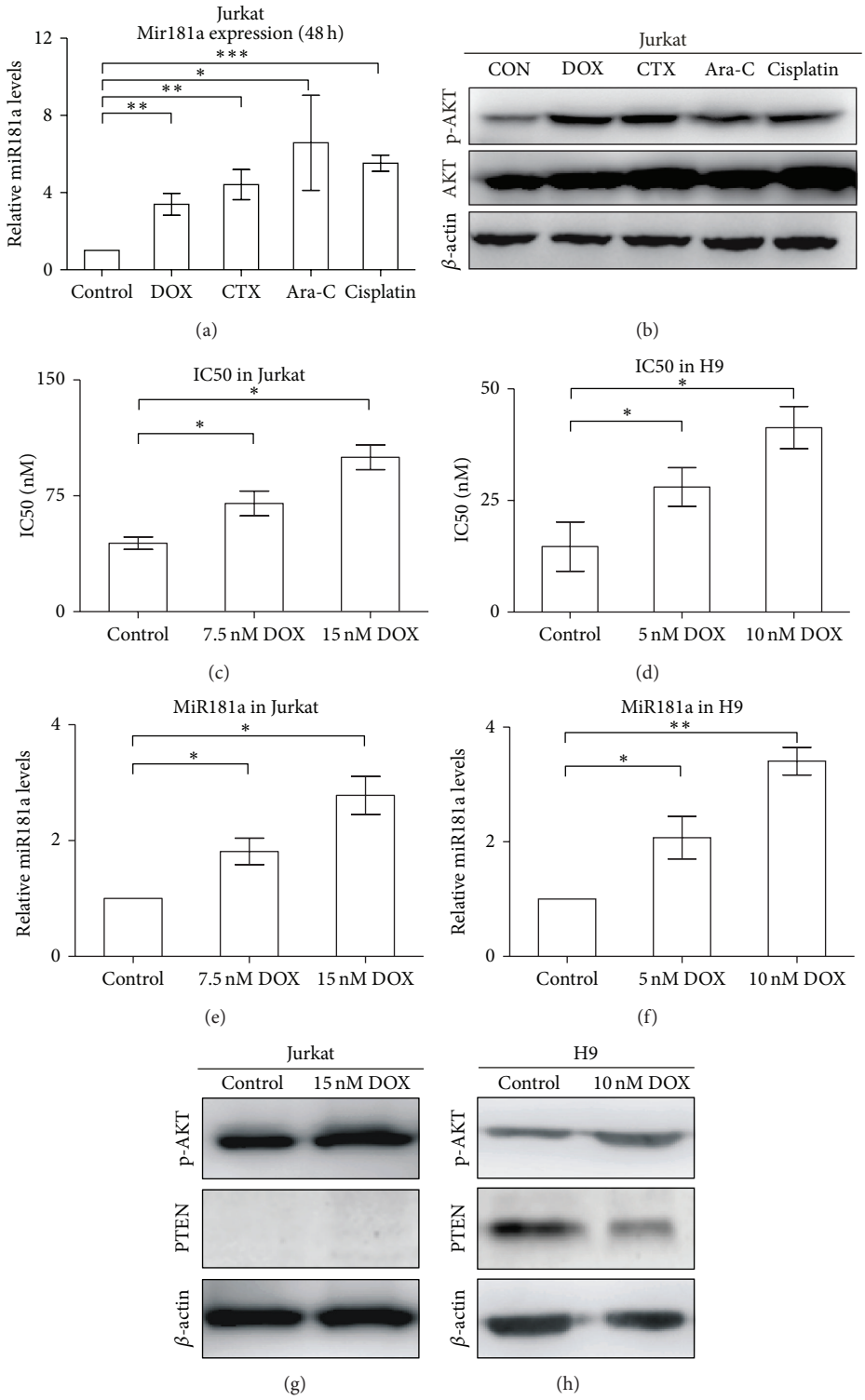

Figure 4: Exposure of T-leukemia/lymphoma cells to chemotherapeutic agents upregulated miR181a expression and resulted in AKT activation. (a) When Jurkat cells were treated with chemotherapeutic agents for 48 h, miR181a expression was significantly increased. CON, untreated; DOX, doxorubicin; CTX, cyclophosphamide; Ara-C, cytarabine. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ comparing with the CON cells. (b) In accordance with miR181a upregulation, increased AKT phosphorylation was observed by western blot, while the total protein level remained constant. (c) and (d) IC50 of doxorubicin was significantly increased in doxorubicin-resistant Jurkat (c) and H9 (d) cells, which were exposed to doxorubicin for 3 weeks. (e) and (f) MiR181a expression was significantly increased in doxorubicin-resistant Jurkat (e) and H9 (f) cells. CON, untreated; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$ comparing with the CON cells. (g) and (h) P-AKT expression was significantly increased in doxorubicin-resistant Jurkat (g) and H9 (h) cells.

Table 2: Cross-resistance of doxorubicin-resistant cells to other chemotherapeutic agents.

Agents	IC50			IC50		
	Jurkat	Doxorubicin-resistant Jurkat	P value	H9	Doxorubicin-resistant H9	P value
Cisplatin (uM)	6.2 ± 0.3	9.0 ± 0.4	0.0039	5.7 ± 0.3	8.5 ± 0.5	0.0067
Cyclophosphamide (mM)	3.3 ± 0.2	4.8 ± 0.2	0.0056	2.4 ± 0.2	3.9 ± 0.3	0.0106

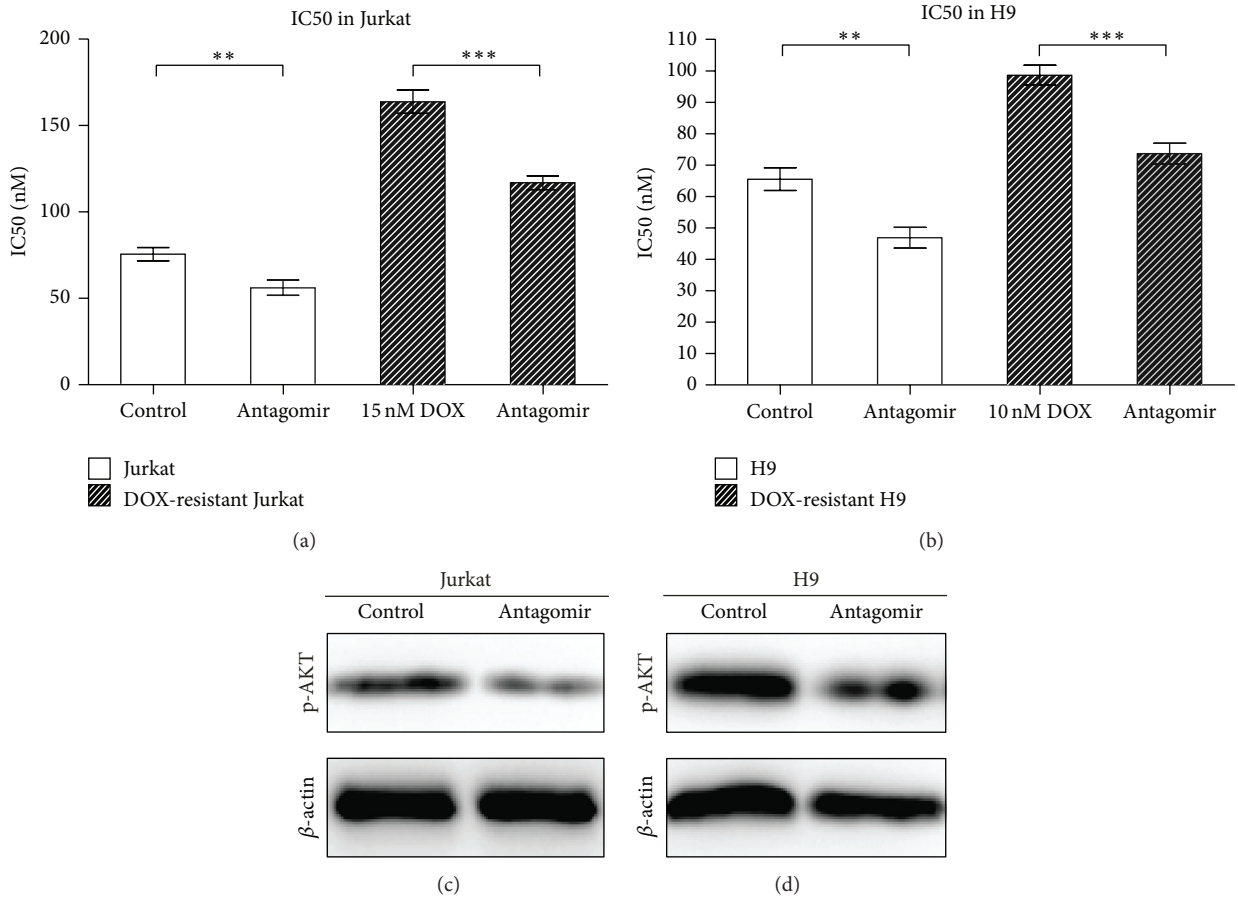

Figure 5: Specific inhibition of miR181a expression could increase T-leukemia/lymphoma cells and resistant sublines sensitivity to doxorubicin, along with p-AKT downregulation. (a) and (b) IC50 of doxorubicin was significantly decreased in antagomir-treated Jurkat (a) and H9 (b) cells. The DOX-resistant Jurkat and H9 cells were exposed to doxorubicin for 6 weeks. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$ comparing with the control cells. (c) and (d) Decreased AKT phosphorylation was found in miR181a antagomir-treated Jurkat (c) and H9 (d) cells.

in T-leukemia/lymphoma. This was consistent with previous reports in B-cell lymphoma that high expression of miR181 could lead to decreasing proapoptotic protein Bim and increasing resistance to chemotherapy [23]. AKT is a key tuning point in tumor cell growth and chemosensitivity [24, 25]. MiR181 is a central regulator of PI3K pathway, since miR181a/b-deficient mice showed severe defects in lymphoid development and T-cell homeostasis associated with impaired PI3K/AKT cascade [8]. As mechanism of action, miR181 targets PTPN, DUSP5, and DUSP6, resulting in PI3K/AKT activation and tumorigenesis in murine T-cell leukemia [7, 8]. Our results showed that ectopic expression of miR181a leads to AKT phosphorylation, enhancing cell proliferation and inducing cell resistance to chemotherapy in T-cell leukemia/lymphoma. This correlation of miR181a overexpression with AKT activation was observed not only in cell lines but also in primary tumor samples of patients with T-leukemia/lymphoma. Apart from primary chemoresistance, acquired chemoresistance is also an important factor of treatment failure. MiR181a expression of Jurkat cells was significantly upregulated after exposure to chemotherapeutic agents and linked to increased AKT phosphorylation. Meanwhile,

Table 3: Inhibition miR181a expression sensitized T-leukemia/lymphoma cells to chemotherapeutic agents.

Agents	IC50			IC50			IC50			IC50		
	Jurkat control	Jurkat antagomir	*P* value	Doxorubicin-resistant Jurkat control	Doxorubicin-resistant Jurkat antagomir	*P* value	H9 control	H9 antagomir	*P* value	Doxorubicin-resistant H9 control	Doxorubicin-resistant H9 antagomir	*P* value
Cisplatin (uM)	6.6 ± 0.2	5.1 ± 0.1	0.0014	9.0 ± 0.4	7.0 ± 0.4	0.0215	5.7 ± 0.3	4.4 ± 0.2	0.0264	8.5 ± 0.5	6.0 ± 0.5	0.0191
Cyclophosphamide (mM)	3.3 ± 0.2	1.6 ± 0.2	0.0018	4.8 ± 0.2	3.6 ± 0.2	0.0214	2.4 ± 0.2	0.9 ± 0.1	0.0019	3.9 ± 0.3	2.5 ± 0.2	0.0134

isogenic doxorubicin-resistant cell lines were developed, which were resistant to doxorubicin and had cross-resistance to other chemotherapeutic drugs. The relative resistance to chemotherapeutic agents was along with increased miR181a expression and subsequent AKT activation, further confirming that miR181a induced AKT activation and contributed to chemoresistance in T-cell leukemia/lymphoma.

5. Conclusions

MiR181a was involved in T-cell leukemia/lymphoma through modulation of AKT pathway. Functioned as a critical regulator of chemosensitivity, miR181a could thus be a promising therapeutic target in treating T-cell malignancies resistant to chemotherapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Zi-Xun Yan, Zhong Zheng, Wen Xue, and Ming-Zhe Zhao contributed equally to this paper.

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Clinical Study

Clinical Safety and Immunogenicity of Tumor-Targeted, Plant-Made Id-KLH Conjugate Vaccines for Follicular Lymphoma

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We report the first evaluation of plant-made conjugate vaccines for targeted treatment of B-cell follicular lymphoma (FL) in a Phase I safety and immunogenicity clinical study. Each recombinant personalized immunogen consisted of a tumor-derived, plant-produced idiotypic antibody (Ab) hybrid comprising the hypervariable regions of the tumor-associated light and heavy Ab chains, genetically grafted onto a common human IgG1 scaffold. Each immunogen was produced in *Nicotiana benthamiana* plants using twin magnICON vectors expressing the light and heavy chains of the idiotypic Ab. Each purified Ab was chemically linked to the carrier protein keyhole limpet hemocyanin (KLH) to form a conjugate vaccine. The vaccines were administered to FL patients over a series of ≥6 subcutaneous injections in conjunction with the adjuvant Leukine (GM-CSF). The 27 patients enrolled in the study had previously received non-anti-CD20 cytoreductive therapy followed by ≥4 months of immune recovery prior to first vaccination. Of 11 patients who became evaluable at study conclusion, 82% (9/11) displayed a vaccine-induced, idiotype-specific cellular and/or humoral immune response. No patients showed serious adverse events (SAE) related to vaccination. The fully scalable plant-based manufacturing process yields safe and immunogenic personalized FL vaccines that can be produced within weeks of obtaining patient biopsies.

1. Introduction

Non-Hodgkin's lymphoma (NHL) is the sixth most common malignancy occurring in adults in the United States with a doubling of incidence since the 1970s [1]; in 2014, more than 70,000 new cases of NHL were diagnosed in the United States alone [2]. The worldwide incidence of NHL is estimated to be 6.1 per 100,000 in males and 4.0 per 100,000 in females with a mortality rate of 3.5 and 2.3 per 100,000 in males and females, respectively [1]. In the West, over 90% of NHL

is comprised of B-cell lymphomas and the most common indolent B-cell lymphoma is follicular lymphoma (FL), which comprises approximately 22% of all B-cell lymphomas [3]. FL is an indolent yet incurable malignancy [4]. The typical clinical course of FL often spans over eight to twelve years during which time multiple lines of therapy can induce remission. Although successfully treated with chemotherapy given with or without rituximab, recurrence is common with each remission being progressively shorter in duration. The use of prolonged administration of rituximab improves event-free survival but not overall survival, and retreatment with rituximab at progression yields the same benefit as use of rituximab as maintenance therapy [5]. Hence, sustaining remission to improve overall survival has been difficult to achieve with available therapies. Strategies to improve the outcome of patients with FL are needed.

Follicular NHL is a clonal B-cell malignancy that expresses a unique idiotype "Id": the antigen-binding site (variable region in light and heavy chains) in the antibody produced by the B-cell clone. The idiotype of a particular B-cell lymphoma has no known ligand but rather represents a tumor-specific antigen and, as such, presents a plausible target for clinical lymphoma treatment [6]. Taking advantage of this molecular targeting feature, experimental therapeutic vaccines against B-cell NHL have been designed to induce idiotype-specific immune responses to control the malignant clone specifically, without impact on the nonmalignant B-cell repertoire. Such tumor-targeted therapeutic vaccines have been produced using a number of different technologies, including human-mouse heteromyelomas [6–9], baculovirus-insect cell culture [10], and transient expression of the idiotype in green plants [11, 12], including our own prior work with agroinfection [13]. The idiotypic vaccines produced through these various platforms have been extensively studied in the clinic for more than 25 years, and, as reported in the above-referenced human and animal studies, proven to be safe and well tolerated [14].

Regardless of production platform, an individualized, custom-made idiotype vaccine for targeted therapy must be produced for each patient [15]. Therefore, any manufacturing method aimed at commercial implementation must be at a minimum (1) flexible, to express a multitude of individual patient-derived idiotypes; (2) robust, to accommodate heterogeneous physicochemical properties of the immunogen; (3) high-yielding, to provide minimum workable expression levels and recovery efficiencies; (4) rapid, to enable provision of vaccines to clinical centers quickly; (5) cost-effective, to make vaccination cost-competitive with current standards of care; and (6) quality compliant, to enable licensure of the vaccine product in multiple regulatory jurisdictions.

It may seem counterintuitive to consider the use of whole green plants as a viable platform relative to cell culture-based idiotype production systems, especially when considering the criteria of speed and the flexibility to manufacture a large number of small amounts of individualized proteins. However, in prior work some of us had shown that transient viral vectors could produce idiotype NHL immunogens in green plants rapidly and that the resultant vaccines met clinical safety and immunogenicity criteria [11, 12, 16]. The landmark study reported by McCormick et al. [12] represented the first plant-made vaccines to be parenterally administered to human subjects in a clinical trial under FDA IND. Notwithstanding the scientific significance of that study on plant-made NHL vaccines, the viral vectors used to manufacture the vaccines produced single-chain antibody fragments (scFv) representing the idiotype. Expression yields were highly variable [11, 13]; furthermore, the lack of a common molecular "handle" made standardized purification of each individual protein impossible and hence the system presented challenges in scalability, efficiency, and costs.

In the present study we demonstrate for the first time that a complex conjugate vaccine for targeted FL treatment can be produced in plants quickly using a fully scalable platform and in compliance with cGMP guidelines. We further demonstrate that such vaccines are safe and well tolerated and can induce tumor idiotype-specific humoral and cellular immune responses in a clinical study with FL patients. This is the first report of successful plant-based production of whole-antibody idiotype immunogens and their performance when administered to patients in a clinical study.

Vaccines for the present clinical study were produced using Icon Genetics' magnICON technology, a plant-based protein expression system that allows rapid and high-yield expression of exogenous proteins but does not result in genetic transformation of the plant; the advantages of magnICON vectors were reviewed by Gleba et al. [17]. Two plant viral expression vectors (one each for the light and heavy chain) encoding the tumor-derived idiotype immunoglobulin were used to transform *Agrobacterium tumefaciens*, which was in turn used to cotransfect the leaves of the plant host *Nicotiana benthamiana*, a close relative of tobacco (*N. tabacum*), using a vacuum infiltration step [17–19]. This transfection step induces the protein expression machinery of the host plant to express high levels of the tumor-derived immunoglobulin (idiotype). The transfected cells in the leaves of these plants are the equivalent to an individualized manufacturing cell line. Upon harvesting and extraction of the leaves and following standard immunoglobulin purification processes (i.e., protein A affinity chromatography, described in Bendandi et al. 2010 [13]), each tumor-derived immunoglobulin was chemically linked to the immunogenic carrier protein keyhole limpet hemocyanin (KLH). The manufacturing process was conducted in compliance with US FDA cGMP guidance. After quality release testing, the vaccines were shipped to the clinical trial sites for patient immunization in a Phase I safety and immunogenicity study.

This Phase I study's primary clinical goal was to demonstrate the safety of the recombinant autologous vaccines manufactured by the magnICON plant-expression technology (also known as "magnifection") in patients who achieved a remission with a non-anti-CD20 based chemotherapy regimen. Secondary objectives were to evaluate idiotype-specific cellular and humoral immune responses to vaccination. In addition to assessing the immunogenicity of the vaccines, the main nonclinical goal of this study was to evaluate the performance and prospects of the magnifection process as a scalable, cost-efficient manufacturing platform for potential commercial implementation.

2. Materials and Methods

2.1. Vaccine Manufacturing. The manufacturing of idiotype vaccines in plants using magnifection technology was described previously [13] and is summarized below. Manufacturing was conducted by Icon Genetics GmbH (Halle/Saale, Germany) in compliance with FDA cGMP guidelines [20, 21].

The antigen used in these studies comprises an Id-containing IgG obtained by genetically fusing the variable region of the patient's tumor-specific Id with a generic constant domain of a human IgG1, regardless of the original tumor isotype (IgG or IgM; antigens of the IgA isotype were not included in this study). The variable light chain (V_L) is fused to a generic kappa or lambda light-chain constant domain, depending on whether the patient's tumor-specific Ig contained a kappa or a lambda light chain. For the magnifection process, the variable regions of the Id were then subcloned in magnICON vectors (Icon Genetics, Halle, Germany) containing a plant signal peptide (rice or bean α-amylase) and a codon-optimized Ig constant region. The variable regions of the heavy and light chains were subcloned in both tobacco mosaic virus (TMV) and potato virus X (PVX) vectors, so that infiltration can be carried out with the heavy chain expressed in TMV and the light chain in PVX and vice versa. The TMV and PVX expression vectors were transformed into the industrial *Agrobacterium* strain ICF 320, a disarmed, auxotrophic derivative ($\Delta cysK_a$, $\Delta cysK_b$, and $\Delta thiG$) of *Agrobacterium tumefaciens* strain C58, which was generated for this process. Both construct combinations were then delivered via *Agrobacterium* into *Nicotiana benthamiana* [18, 22] to test for the best possible combination of expression of the Id (data not shown). The above procedures resulted in successful expression of 21 patient-derived Igs of 21 attempted. Only one Ig could not be purified in sufficient quantity, so the final Ig manufacturing success rate was 20 Ig purified of 21 attempted.

A highly robust and reliable protocol for Ig purification based on protein A affinity capture was developed for molecules produced in this study. For Ig purification, 5-kg batches of green biomass were homogenized and acidified. Lowering the pH of the plant homogenate to <5.1, holding, and subsequently raising the pH to 8.5 followed by filtration enabled the removal of many highly abundant host cell proteins, for example, rubisco and larger debris. The resulting crude extract was suitable for subsequent chromatography. After protein A affinity capture, the Ig-containing eluate was further purified by membrane adsorption chromatography. This protocol enabled the reliable purification of Igs for vaccine manufacturing.

Each Id MAb was subjected to stringent quality control (QC) analyses, including appearance (visual), total protein (A280), purity (CGE and SEC-UV), identity (MW by SEC-LS; tryptic MALDI analysis of light and heavy chain fragments), endotoxin (harmonized method), residual DNA (threshold method), and sterility (harmonized method). MALDI MS analysis of purified IgG molecules cloned from all patients on study was used to calculate the differences between theoretical and determined MW caused by glycosylation. Glycosylation was further analyzed by LC-ESI-MS.

MAb immunogens that passed QC release criteria were subjected to KLH conjugation with glutaraldehyde, as described [13]. The conjugate vaccines were subjected to additional testing to meet specification. These vaccines comprised the drug products (final container) that were released for clinical administration.

2.2. Identity of Investigational Product. Genetic material required to produce each vaccine was produced from single-cell suspensions of lymphocytes taken from each patient's excised tissue and produced the same day as tissue procurement. These lymphocytes were sent under temperature-controlled conditions within 24 hours of collection and processing to the vaccine manufacturing facility at Icon Genetics in Germany. The resultant recombinant vaccine was supplied to the clinics in sealed, sterile glass vials.

Each vial of study drug contained 0.5 mg of idiotypic protein conjugated to 0.5 mg KLH in 1.0 mL clear phosphate-buffered saline with up to 0.3 mL overfill. Label information on each vial included batch number comprised of serial batch number and "UPIN" (a Unique Patient Identification Number), Study Number, contents labeled as "Recombinant Idiotypic Vaccine" and "New Drug" for investigational use, concentration of 1 mg/mL, date of manufacture, name of sponsor, and name of clinical research organization (CRO) overseeing study conduct. Study drug was stored frozen at the manufacturing facility until requested by the site. Vaccine vials were shipped with temperature recording at $\leq -50^{\circ}$C on dry ice from the manufacturing facility to sponsor's CRO, which then transported the vials directly to study sites. At each site, study drug was kept at $\leq -50^{\circ}$C until time of administration.

2.3. Study Objectives

2.3.1. Primary. The primary objective of this clinical study was to evaluate the safety and tolerability to the magnICON produced Id vaccines administered with GM-CSF over a 6-cycle vaccination phase, wherein grade 3–5 adverse events are deemed to occur in <17% of patients and to be vaccine-related and unexpected as assessed by the FDA CBER Guidance for Industry for Toxicity Grading Scale in Preventive Vaccine Clinical Trials [23] and NCI-CTCAE version 4.02.

2.3.2. Secondary. The secondary objectives were:

(1) Assessment of humoral idiotype-specific immune response to vaccination defined as ≥40% of subjects developing a humoral immune response;

(2) Assessment of cellular idiotype-specific immune response to vaccination defined as ≥50% of subjects developing a measurable cellular response;

(3) Long-term safety/tolerability to the vaccines up to the conclusion of a 12-cycle vaccination phase, as determined by <17% of patients showing vaccine-related and unexpected grade 3–5 adverse events as assessed by the FDA CBER Guidance for Industry for Toxicity Grading Scale in Preventive Vaccine Clinical Trials and the NCI/CTCAE version 4.02.

2.4. Study Design and Rationale. This study was a single arm, repeated dose, nonrandomized Phase I trial evaluating the safety of the individualized recombinant idiotypic vaccines administered to patients in remission after treatment for relapsed follicular non-Hodgkin's lymphoma.

2.4.1. Subject Enrollment, Inclusion, and Exclusion Criteria. Enrollment was open to patients with histologically proven follicular lymphoma relapsed after prior therapy or transformed follicular lymphoma relapsed after prior anthracycline therapy. Other key eligibility criteria at initial screening consisted of the following:

(1) The tumor cells must express either an IgM or an IgG on their surface, but no IgA or lack of surface immunoglobulin.

(2) Bone marrow involvement, known or unknown, is allowed.

(3) Age > 18 years.

(4) ECOG performance status of 0–2.

(5) Life expectancy of at least 12 months.

(6) Presence of at least a 2×2 cm in diameter single lymph node or equivalent volume of nodes accessible by physical examination for excision, for histological confirmation of diagnosis, and for manufacture of the vaccine.

(7) No exposure to rituximab or anti-CD-20 directed therapy within 4 months prior to enrollment.

Additional exclusion criteria at initial screening or at time of initiation of vaccination included:

(1) Development of intercurrent illness such that at the discretion of the investigator proceeding with chemotherapy or vaccination phases of the protocol would be detrimental;

(2) Uncontrolled hypertension despite optimal treatment;

(3) History of cardiac disease;

(4) History of HIV (+), HBs antigen (+), or HCV (+);

(5) Active clinically serious infections (>grade 2 NCI-CTC version 4.02).

2.4.2. Study Design. The trial evaluated one-dose level of vaccine with no dose modification. The primary objective defined for the study was to evaluate safety and tolerability of the magnICON produced idiotype vaccine administered with GM-CSF over a ≥6-cycle vaccination phase when given to patients in complete remission (CR) or very-good PR (near-CR) following non-rituximab (non-anti-CD20) containing salvage chemotherapy for relapsed or transformed follicular lymphoma. Other objectives included immune response to the idiotype vaccine and long-term safety for patients who complete 12 doses of vaccine. The trial scheme is shown in Figure 1.

The trial design sought to identify and ultimately evaluate vaccine safety and immunogenicity in a relatively uniform group of patients. Thus, a single histologic subtype (follicular lymphoma), disease status (relapsed setting), and salvage therapy treatment (cytotoxic therapy with no anti-CD20 therapy) were required for participation. Avoidance of anti-CD20 therapy for at least 12 months prior to study drug exposure was incorporated into the study design. This was achieved by restricting eligibility to patients who had not received anti-CD20 therapy within 4 months of enrollment, using non-anti-CD20 containing salvage therapy for at least 4 months and incorporation of a 4-month observation phase prior to the start of study drug. This design was elected in an attempt to optimize each patient's ability to mount an immune response to study drug by avoiding the known long-lasting immunosuppressive effects of anti-CD20 therapy.

2.4.3. Subject Withdrawal Criteria. Subjects could withdraw from the study at any time at their own request or be removed if, in the investigator's or sponsor's opinion, continuation in the study would be detrimental to the subject's well-being. Protocol specified reasons for study discontinuation were:

(1) Patients not achieving a CR or PR after completion of salvage chemotherapy phase of this study;

(2) Substantial noncompliance with the requirements of the study as defined by the coordinating investigator;

(3) Patients with laboratory test results consistent with pregnancy. The pregnancy will be followed until delivery or resolution via the Pregnancy Monitoring Form. Pregnancy will be reported along the same time lines as a serious adverse event;

(4) Use of illicit drugs or other substances that may, in the opinion of the investigator, have a reasonable chance of contributing to toxicity or otherwise skewing results;

(5) Development of an intercurrent illness or situation which would, in the judgment of the investigator, interfere with the safety of the study subject on study therapy or affect assessments of clinical status and study endpoints to a significant degree;

(6) The development of a second cancer;

(7) Patient who is lost to follow-up;

(8) Patient's death.

2.5. Study Sites. Patient recruitment and vaccine administration were performed at two sites in Dallas, Texas, United States, namely, The Simmons Cancer Center of the University of Texas Southwestern Medical Center and the Baylor Sammons Cancer Center. The conduct of this study at these sites adhered to ethical guidelines for research on human subjects in accordance with the Declaration of Helsinki (1964). The study was conducted with the understanding and the consent of each subject who elected to participate in the research (see next section). Each site's Institutional Review Board (Ethical Committee) reviewed and approved all aspects of the research prior to the enrollment and treatment of any subject.

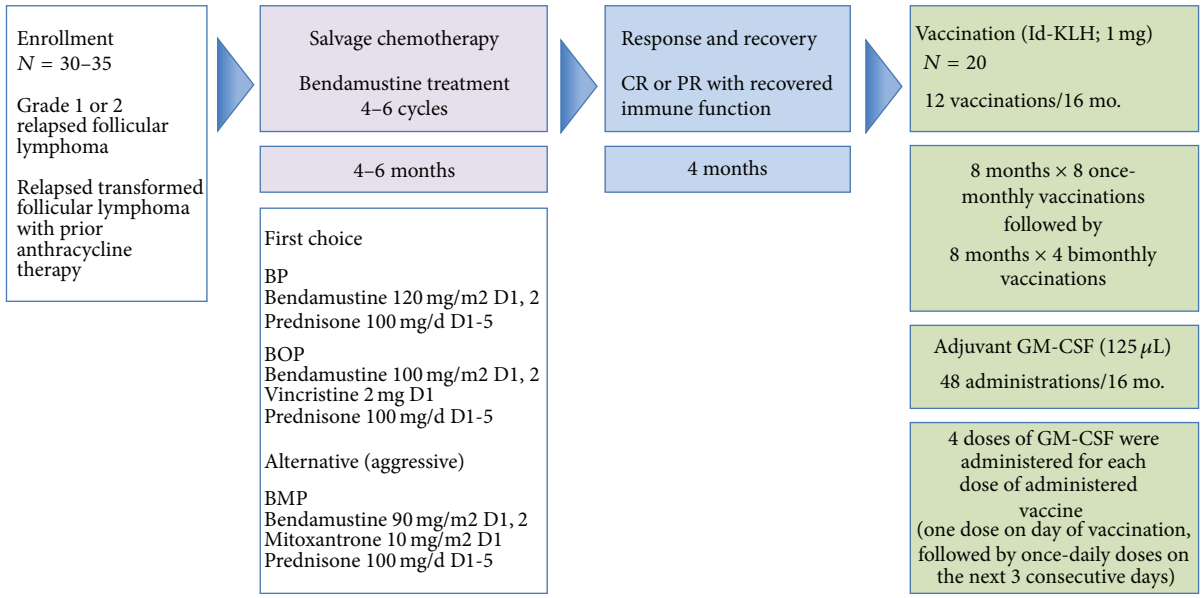

Figure 1: Study design summary: Phase I study of an autologous vaccine manufactured in plants by magnICON technology for the treatment of patients with relapsed or transformed follicular lymphoma.

2.6. Clinical Study Demographics. A summary of the demographic characteristics of patients enrolled on the trial is presented in Table 1. A majority of patients were male of age ≥50, as expected for the diagnosis of follicular lymphoma where the mean age of onset is 60 [1]. Although the trial sought and was available to persons of all ethnic groups, specific recruitment of minority groups was not pursued.

2.7. Subject Information and Consent. Prior to the commencement of any study specific procedures, including the study required lymph node excision, the PI or designee was responsible for proper informed consent and for obtaining a signed version of the ICF from each subject or legal representative.

The unique study design included at least an 8-month interval from study consent and enrollment prior to the start of investigational agent. Because of this intentional delay in consent until start of study drug, reconsent using the same consent form was required at a second screening step performed prior to start of study drug administration.

2.8. Clinical Study Conduct. This program was conducted in compliance with IND guidance under the jurisdiction of the Center for Biologics Evaluation and Research (CBER), US Food and Drug Administration (FDA), and was conducted in compliance with clinical trials registration guidelines. This study was registered with ClinicalTrials.gov in November 2009 with identifier NCT01022255. In addition, the clinical study was conducted in compliance with consolidated ICH guidelines for Good Clinical Practice [24] and all aspects of the clinical work were doubly monitored by each center's Institutional Review Board (IRB) and by an independent Clinical Research Organization (CRO) on behalf of the study's sponsor.

Table 1: Demographic information of enrolled patients ($N = 27$).

Category	Number of subjects
Age group	
18–33	0
34–49	3
50–65	13
>65	11
Gender	
Male	18
Female	9
Race	
Caucasian	24
Asian	0
Hispanic	2
African American	1
Other	0

For operational purposes the trial was defined by the following phases.

2.8.1. Screening Phase. This phase of the study included obtaining patient consent, performing screening tests, obtaining patient-specific tissue samples by outpatient surgical biopsy, and determining eligibility for the study based on screening test results and histologic information from tissue obtained at surgical biopsy. Adequate tissue was obtained at biopsy for autologous vaccine production.

2.8.2. Chemotherapy Phase. As the effects of chemotherapy were not the focus of this study and the agents were FDA approved, this phase of treatment could be given by a study

investigator or by a noninvestigator oncologist in communication with a study investigator. Because no investigational agents were administered and chemotherapy was given per standard dose and schedule, data collection was limited and safety reporting was not required for this phase of the trial.

After tumor excision, each subject fulfilling screening requirements received a salvage chemotherapy regimen with bendamustine (Treanda), in combination with other approved chemotherapeutic agents, to debulk the tumor and induce a measurable partial (PR) or complete remission (CR). Anti-CD20 therapy was not allowed. Bendamustine-based chemotherapy was selected because in disease refractory to rituximab and alkylator agents, bendamustine demonstrated an overall response rate of 70–90% [25–27] with complete response rates approaching 35%. For this trial the regimen of bendamustine, vincristine, and prednisone (BOP) or bendamustine and prednisone (BP) were defined as first choice options for salvage chemotherapy to be given only for 4–6 cycles, as prolonged chemotherapy was felt to compromise immunologic recovery. Patients were restaged after cycle 4 of chemotherapy and those not responding optimally to BP (or BOP) could withdraw from the study or be given 2 cycles of bendamustine, mitoxantrone, and prednisone (BMP) as specified by protocol to try to achieve the required CR/very good PR at the end of the salvage chemotherapy phase.

Patients who progressed, had stable disease, or had less than a very good PR as best response to 6 cycles of chemotherapy were taken off study.

2.8.3. Observation Phase. After conclusion of the chemotherapy phase, the patient was then monitored for general health and immune recovery for 4 months. Patients were clinically reassessed with scans and laboratory analyses at the end of the observation phase. If disease was in stable CR/very good PR at this assessment, the patient was then reconsented and started on study drug.

2.8.4. Vaccination Phase. During this last phase of the study, patients received the study drug given by monthly subcutaneous injection × 8, followed by bimonthly injection × 4, for a total maximum of 12 injections over 16 months.

2.9. Study Site Logistics. The sponsor of this study was Bayer Innovation GmbH, a subsidiary of Bayer AG, Leverkusen, Germany. Eligible patients were consented at two clinical study centers and excisional biopsies of each patient's tumor were obtained by a single study designated surgeon at a single study designated outpatient surgery center located in Dallas, Texas, United States. Within 2 hours of excision, tissue was reviewed for histology and isotype determination. If these qualifications did not meet study eligibility the patient was categorized as a screen failure. Otherwise aliquots of these tissue samples were shipped within 24 hours via air courier under temperature-controlled conditions to Icon Genetics' manufacturing facility in Halle, Germany, and to University of Navarra's clinical analytics facility in Pamplona, Spain. The vaccines were manufactured in Germany and shipped under temperature-controlled conditions to the CRO in Dallas, who after verifying sample identity and quality control documentation, transported the vaccines under temperature controlled conditions to the investigational pharmacy of the clinical centers for storage and subsequent administration to patients. During the study, patient samples of serum and plasma as well as peripheral blood mononuclear cells were obtained at each protocol designated sampling point and stored frozen. These samples were then batch-shipped under temperature-controlled conditions to the University of Navarra's Laboratory of Immunology, for vaccine immune response analysis.

2.10. Vaccine Administration. At the time of each scheduled administration, one vial labeled with the verified patient identifier (UPIN) was removed from the investigational pharmacy freezer and set aside at room temperature to thaw prior to injection. One mL of vaccine solution was drawn into a syringe and administered within 30 minutes of thawing at the selected subcutaneous injection site. The 1 mL dose contained 1 mg protein comprised of 0.5 mg Ig conjugated to 0.5 mg KLH. This Phase I trial did not attempt to determine an optimal dose; the 1 mg dose was in the range of 0.5 to 2.0 mg used in other trials with similar compositions [6, 8, 10, 12]. The study focused instead on determining safety, tolerability, and immunogenicity of the plant-produced product and its impurities.

As in prior FL vaccine studies, GM-CSF was added as an adjuvant to optimize immunologic responsiveness. GM-CSF at 125 μL was administered at the injection site once on the day of vaccination and once daily for the next 3 days following vaccine administration. Thus, each subject received 4 doses of GM-CSF for every dose of idiotypic vaccine.

A planned maximum of 12 vaccinations was to be given over 16 months for patients who remained stable during the vaccination phase of the study. Vaccines were given once every 28 days ± 3 days for the first 8 doses then every 56 days ± 3 days for the last 4 doses. Study subjects could withdraw from the study at any time or were removed for recurrent disease requiring therapy that appeared during the vaccination phase.

2.11. Immune Responses to Vaccines. The immunological responses to the study drug were assessed by laboratory measurement of humoral and cellular immune responses from samples taken at baseline and monthly just prior to each vaccination, throughout the vaccination period. In particular, idiotype-specific humoral immune responses were assessed in both pre- and post-vaccine sera (via ELISA). Humoral immune responses were also assessed against the immunogenic carrier protein KLH, which is a component of these conjugate idiotypic vaccines, as an indicator of the patients' immune status since KLH is a strong immunogen that typically yields a positive response. Cell Mediated Immunity assays (CMI), such as ELISpot, flow cytometry, cellular proliferation, and/or multiplex immunological assays were performed as applicable as primary or confirmatory methods to assess the overall immune response, as well as specific recognition of each patient's idiotype and tumor-specific antigens. Peripheral blood mononuclear cells were collected from each patient and analyzed for immune status to help

correlate responsiveness to vaccination. Positive and negative response criteria for each patient followed convention, as described by Inogés et al. 2006 [8].

A successful immunologic endpoint of this study was defined as (a) greater than 40% of subjects developing humoral immune responses after receipt of vaccine 6, or (b) greater than 50% of subjects developing cellular immune response after receipt of vaccine 6.

2.12. Immune Responses to Plant Glycans. All recombinant idiotypic antigens used in this study were glycoproteins and comprised IgG that contained oligomannosidic as well as plant-type glycans, the latter including vacuolar and secretory type structures (data not shown). Patients' pre- and post-immune sera were tested by ELISA for binding to plant glycosylated glycoproteins. Analyses were done in triplicate using vaccinated patient sera diluted 1 : 2000. Two antigens were used as controls, horseradish peroxidase (a glycosylated plant enzyme), and a plant-produced idiotype. For patient U001, the control idiotype was U011; for all other patients, the control idiotype was U001. Detection of serum binding was with alkaline phosphatase-labeled antisera. For ELISA plates coated with horseradish peroxidase, an anti-human IgG Fc antiserum was used. For ELISA plates coated with control idiotype U001, an anti-human kappa antiserum was used, and for control idiotype U011, an anti-human lambda antiserum was used. The presence of plant glycans on ELISA plates coated with horseradish peroxidase or control idiotypes was confirmed using a rabbit antiserum raised against *Phaseolus vulgaris* lectin that is known to bind to plant complex glycans. Detection was with anti-rabbit IgG antiserum labeled with alkaline phosphatase.

2.13. Statistical/Analytical Issues. The study population was too small to apply statistical analyses, as defined per protocol. Specific analytical issues (e.g., availability of lymphoid samples; minimum lymphocyte counts; success rate in vaccine production; reproducibility) are discussed in the following sections.

3. Results and Discussion

3.1. Manufacturability of Vaccines. Each personalized conjugate vaccine was manufactured and released within approximately 12 weeks of biopsy material reaching the manufacturing facility. This time reflects patient-specific sequence determination, expression of the idiotypic Ig (tumor-specific antigen) in plants and its purification, which required 2 weeks, and the time required for KLH conjugation of each antigen, purification, and quality control analyses for cGMP-compliant vaccine release.

The plant-based system used to manufacture the Ig for this study was capable of expressing all (21 of 21) idiotypic antigens scheduled for cloning. Of the 22 biopsies that were received at the manufacturing facility, one was found to code only for the Ig heavy chain and was rejected because it did not meet the idiotype specification; of the remainder, all 21 could be expressed *in planta*. Of those 21, only 1 could not be purified in sufficient quantity to develop a vaccine. In five instances total, patient biopsy material was difficult to obtain, contained insufficient cells from which to clone the idiotype, or was unavailable for other technical reasons and hence no vaccines were produced. These constituted "screen failures" and were not counted in the vaccine manufacturing tally (Table 4).

An overview of the unit operations of the NHL vaccine manufacturing process employed is presented in Figure 2.

The speed, efficiency, and yield achieved with the magnICON expression system in producing patient-customized clinical materials for this study compared favorably with results we reported during developmental studies [13], in which we showed that all attempted vaccine antigens (22 of 22), including 20 human lymphoma-derived Ig and 2 murine lymphoma-derived Ig, were successfully produced. In the current study, 20 of the 21 Ig were successfully purified and released with cGMP compliance in sufficient yield to enable vaccine product manufacturing.

3.2. Patients Receiving Study Drug. The study initially planned to enroll approximately 30–35 patients to undergo chemotherapy (Figure 1) and to ultimately select 20 patients to receive vaccination, expecting that approximately 10 patients would drop from study due to disease progression, removal of consent, health complications not related to therapy, and other reasons. In actuality 27 candidates were screened, consented, and were enrolled. Five patients who initially met enrollment criteria failed laboratory screening tests specifically in the setting of node excision. Fourteen patients did not receive any study drug due to health reasons or progression prior to study drug administration. At the conclusion of the study, 135 vaccine administrations were given to 15 patients who had received at least one dose of vaccine, and 8 patients completed all 12 planned vaccinations, as summarized in Table 2. Eleven patients of the 15 patients receiving study drug received a minimum of 6 vaccinations and became evaluable for immune response to vaccination.

3.3. Safety and Tolerability

3.3.1. Chemotherapy Phase. In this study, non-rituximab-based salvage chemotherapy was administered prior to initiation of vaccination. Adverse events and serious adverse events (AE/SAE) observed during the chemotherapy phase of the trial were not unexpected and were typical for those observed with the drugs used (data not shown). This trial was not designed to assess the efficacy of approved drugs for salvage therapy; nevertheless a valuable metric was derived with respect to immune depletion and its potential impact on immune response to vaccination. Most patients receiving vaccination were lymphopenic to various extents even after 4 months of recovery after receiving the last dose of chemotherapy (Table 4).

3.3.2. Vaccination Phase. Adverse events were reported as defined by the 2007 CBER FDA grading scale for preventative vaccines. If toxicity was identified but not described in the CBER grading scale, the event was graded per the NCI CTCAE v4.02. Adverse events reported originally as a lower

Table 2: Patient recruitment summary.

Planned	Screened/consented	Screen failure	Off study	Vaccinated	Completed study
30	27	5	14	15	8

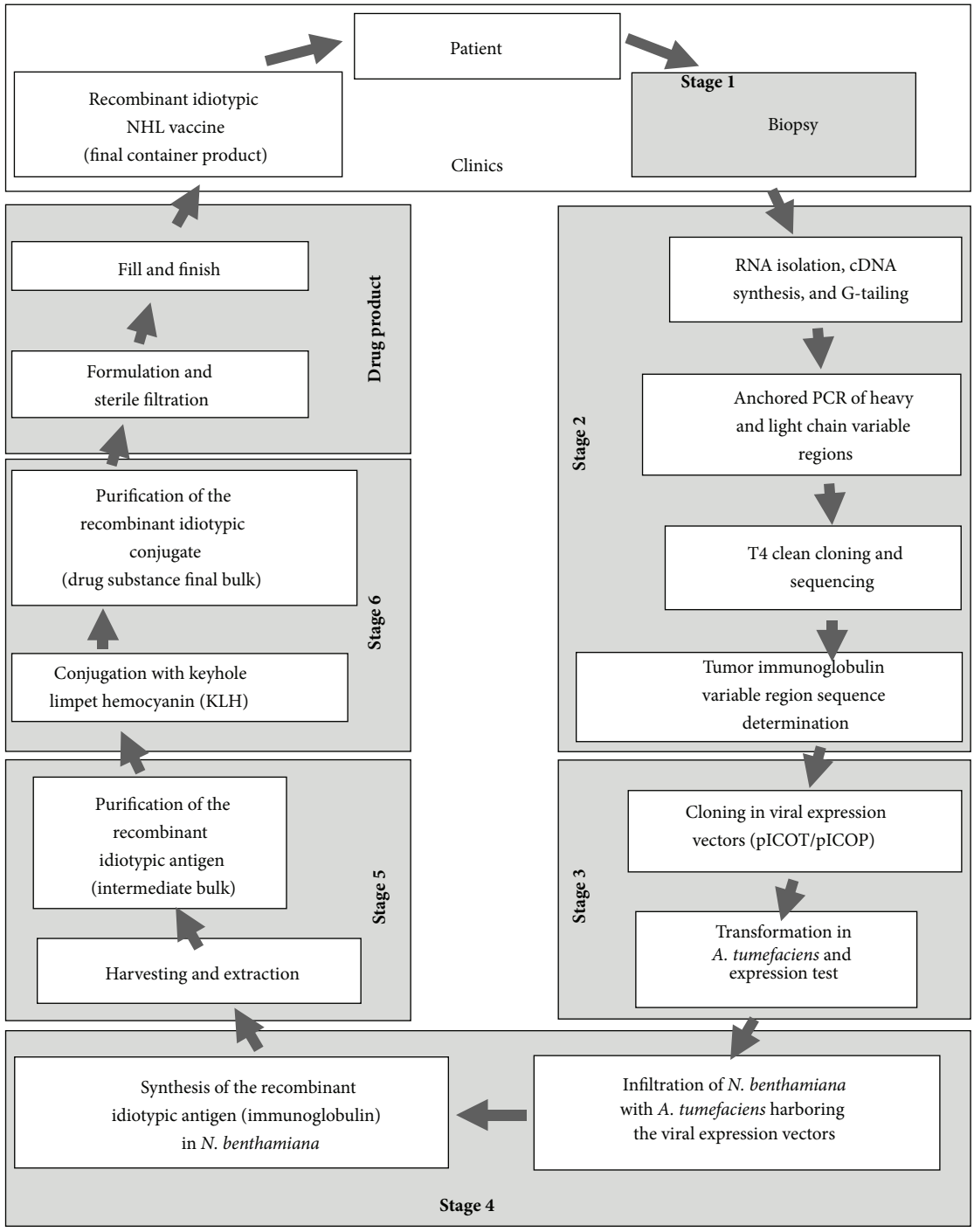

Figure 2: Individualized NHL vaccine manufacturing process overview.

Table 3: Summary of adverse events reported during the vaccination phase of the study.

		Maximum CBER/NCI CTC grade by patient			
	Total (related)	Grade 1	Grade 2	Grade 3	Grade 4
Local injection reactions	**109** (96)	57	36	12	4
Systemic symptoms	**140** (85)	61	71	8	0
Infections	**11** (0)	2	9	0	0
Musculoskeletal	**127** (81)	58	62	7	0
Gastrointestinal	**42** (14)	30	11	1	0
Neurologic	**7** (2)	1	4	2	0
Other	**7** (0)	2	5	0	0
Total	**443 (278)**	**211**	**198**	**30 (20)**	**4 (1)**

Total number of adverse events (AE) by category reported throughout the study regardless of attribution. Numbers in parenthesis indicate AE that were possibly, probably, or definitely related to vaccination. Study safety objectives are defined by the frequency of vaccination-related grade 3–5 AE. No grade 5 AE occurred.

grade and subsequently worsening were captured twice, at the original lower grade and again at the worst grade. Adverse events which improved after initial reporting were captured only once at the original worst grade. Serious and nonserious adverse events were recorded during the vaccination phase of the trial for any patient receiving any dose of vaccine from 1 to 12. A total of 443 systemic and local nonlaboratory AE were reported regardless of attribution with 278 categorized as possibly, probably, or definitely related to vaccination.

These 278 nonlaboratory AE can be further categorized as 182 systemic events and 96 local injection reactions; only 21 total AE were grade 3 or 4 in severity (<7.5% for the entire study population). Grade 3 or 4 AE were seen in only 3 patients and only 21 reactions were considered related to study drug.

Table 3 is a summary of adverse events reported throughout the study.

Related grade 3 and 4 adverse events occurred as local injection site reactions, systemic symptoms, and musculoskeletal symptoms. Local injection site reactions related to study drug were reported if occurring within 7 days of study drug administration; related grade 3 reactions consisted of hardness, pain, redness, and swelling. The only grade 4 event definitely related to vaccination, but not unexpected, was an injection site reaction (injection site redness) occurring in one patient. Systemic symptoms related to study drug were all grade 3 and consisted of fatigue, non-cardiac chest pain, and generalized pruritus across 2 patients. One musculoskeletal grade 3 event, possibly related and not expected, was defined as musculoskeletal neck pain. No grade 3 or 4 events were reported as infections, gastrointestinal, or neurologic toxicities.

In this proof-of-concept trial adverse events categorized as related or possibly related to vaccine occurred in only 3 individual patients; thus, characterization of AE by gender, age, or race is not considered illustrative.

It is important to highlight that each vaccine dose was coadministered with 4 doses of the adjuvant GM-CSF. Therefore, a patient receiving the full course of 12 vaccines would also receive 48 injections of GM-CSF. The attribution of toxicities to the vaccine itself versus the GM-CSF administered concomitantly was difficult to separate and the adverse event profile gathered reflects toxicities for both.

In general, the adverse reactions to the vaccines were not unique from those known to be attributable to GM-CSF and can be easily compared to those toxicities summarized in the product monograph for intravenous Leukine when administered to enhance cytologic recovery post autologous and allogeneic stem cell transplant [28]. Side effects occurring in more than 30% of patients receiving GM-CSF include diarrhea, local reactions such as swelling, redness, and tenderness, and systemic reactions such as fatigue and weakness. Less common effects occurring in 10–29% of patients receiving GM-CSF have been reported to be flu-like symptoms (fever, generalized aches and pains, weakness, and fatigue) and edema of hands and feet. All of these same toxicities were reported for patients receiving the magnICON produced idiotype vaccine followed by GM-CSF. A total of five (5) SAE occurred during the vaccine phase of the study, none of which were attributed to vaccine administration.

Taking results obtained during this trial in their totality, we assert that this study met its primary and secondary safety objectives, namely, short-term and long-term safety and tolerability. The primary objective was to document safety and tolerability of the vaccines over a 6-cycle vaccination phase, defined by the incidence of vaccine-related, unexpected grade 3–5 adverse events occurring in <17% of patients receiving 6 vaccinations. In this study, 11 patients received a minimum of 6 vaccinations and became evaluable; thus, fewer than 2 patients ($11 \times 0.17 = 1.87$ patients) were to experience vaccine-related, unexpected grade 3–5 events during the first 6 vaccinations for this vaccine to be considered safe. The primary endpoint of the trial was achieved; in the 11 patients receiving up to 6 vaccinations, grade 3–5 study drug-related and unexpected events occurred in only one patient.

This study also met its secondary objective for long-term safety/tolerability, as determined by the proportion of patients with toxicities as assessed by the NCI/CTCAE version 4.02 grade ≥3 to the magnICON-produced Id vaccine up to the conclusion of a 12-cycle vaccination phase (month 16). No (0) vaccine-related, unexpected grade 3–5 AE were reported in the 8 patients who completed the full course of 12 vaccinations throughout the entire trial.

Table 4: Humoral and cellular responses to vaccination in the eleven evaluable FL patients on study.

Patient UPIN[1]	Tumor class	Number of vaccinations[2]	Immune status (lymphocyte number)	Humoral response[3]				Cellular response[8]	
				KLH[4]	ELISA Plant Id[5]	Hybridoma Id[6]	Flow Cytometry[7]	T-cell proliferation	ELISpot
U001	IgM-lambda	12 of 12	Normal total lymphocyte count Low CD4+ T-cell count	+	+	+	+	+	−
T003	IgM-kappa	12 of 12	Lymphopenic (all subsets)	+	−	−	ND	−	−
T006	IgG-lambda	12 of 12	Normal total lymphocyte count Low CD4+ T-cell count	+	−	ND	ND	+	+
T008	IgM-kappa	7 of 12	Lymphopenic Normal CD8+ T-cell count	+	−	−	ND	−	−
T010	IgG-kappa	9 of 12	Normal total lymphocyte count Low CD4+ T-cell count	−	−	−	ND	+	+
U011	IgM-kappa	12 of 12	Lymphopenic (all T-cell subsets)	+	+	+	+	+	+
U016	IgM-lambda	12 of 12	Lymphopenic (all T-cell subsets)	+	−	−	ND	+	−
U017	IgM-kappa IgG-kappa	12 of 12	Normal total lymphocyte count Low CD4+ T-cell count	+	−	ND	ND	+	+
T021	IgG-lambda	9 of 11	Normal total lymphocyte count Low CD4+ T-cell count Borderline CD19+ B-cell count	−	+	−	−	+	−
T022	IgG-kappa	12 of 12	Normal total lymphocyte count Low CD4+ T-cell count Low CD19+ B-cell count	−	−	−	ND	+	−
U026	IgM-lambda	12 of 12	Lymphopenic (all subsets)	+	−	ND	ND	+	−

+ = Positive response; − = Negative response; ND = Not determined. 1 = Unique Patient Identification Number. 2 = Number of vaccines administered of the maximum 12 vaccines scheduled. 3 = Humoral responses were measured by ELISA and by flow cytometry. 4 = Specific response to KLH by ELISA was included to assess patients' overall immune responsiveness. 5 = Plant-made idiotype administered in the clinical study. 6 = Equivalent Id made in hybridoma to cross-check immune response to plant-made Id. 7 = Flow cytometry was used only as a confirmatory assay for humoral response when positive ELISA responses were measured. 8 = Cellular immune responses were measured by T-cell proliferation and by ELISpot.

3.4. Immune Responses

3.4.1. Overall Findings. While not designed to assess efficacy, this study evaluated immune responses to tumor antigen-specific vaccination with study drug. Serum samples from all 11 patients who received a minimum of 6 vaccinations were subjected to both humoral and cellular immune response analyses. Of the 11 patients evaluated, nine (9) responded to vaccination (82%). Three (3) of those patients (U001, U011, and T021) mounted both idiotype-specific humoral and cellular immune responses; this represents a 27% double-positive response rate to the vaccine. Nine (9) of 11 patients (82%; patients U001, T006, T010, U011, U016, U017, T021, T022, and U026) mounted an idiotype-specific cellular response. No (0 of 11) patients mounted only a humoral response without also mounting a cellular response. Two (2) of the 11 patients failed to respond to vaccination (18% non-responsive).

For comparative purposes in this research, we also developed hybridoma-produced idiotypes as counterparts to the plant-produced immunogens. These hybridoma-produced idiotypes were used to more specifically characterize patient sera for immune reactivity to the plant-produced idiotypes used in vaccination. Reactivity of patient sera to the immunogenic carrier protein KLH, which is conjugated to every idiotypic Ig and is part of the vaccine composition, was also assessed. Our observations from these comparisons are summarized for each patient evaluated.

The overall results obtained are summarized in Table 4. In the table, a successful immunologic response is marked as "+" and a negative response is marked as "−" following convention and criteria described by Inogés et al. 2006 [8] and others [12, 29].

3.4.2. Interpretation of Immune Responses to Idiotypic Antigens. As expected, administration of personalized vaccines to patients with lymphoma produced a range of humoral and cellular responses. The results summarized in Table 4 are interpreted as follows for the 11 evaluable patients on study who successfully received a minimum of 6 vaccine administrations.

(1) Strong Responders. Three of the 11 evaluable subjects, namely, patients U001, U011, and T021, developed strong tumor idiotype-specific humoral and cellular responses (double positive) with two (U001 and U011) showing humoral responses against the carrier protein KLH, which is a component of all the plant-made conjugate vaccines. Patients U001 and U011 received the full course of vaccination (12 vaccines administered of 12 planned). The humoral response in both of these patients was positive not only to the plant-produced idiotype, but also to a corresponding hybridoma-derived idiotype developed only for comparative purposes. While the two idiotypes are identical, they are grafted onto different scaffolds: IgG for the plant-produced Ig, IgM for the hybridoma-produced Ig; the latter being the original, tumor-specific idiotypes derived from IgM-expressing tumor clones for both patients.

Patients U001 and U011 also showed strong cellular responses to their respective idiotypes, with T-cell proliferation being positive at all pertinent time points when activated autologous tumor cells were used as stimulants. ELISpot confirmatory results were negative for U001 and positive for U011. Patient U001 had a normal or close-to-normal total lymphocyte count (except for low CD4+ T cells) both at screening and throughout most of the vaccination calendar. Patient U011 was lymphopenic (all T-cell subsets) throughout most of the vaccination calendar yet both humoral and cellular responses were detected.

Patient T021 received 9 vaccines of 11 scheduled, having missed vaccines 5 and 7 due to missed appointments and vaccine 12 due to progression. Patient T021's idiotype-specific humoral response was positive by ELISA but negative by flow cytometry, and similarly the patient's idiotype-specific cellular response was positive by T-cell proliferation but negative by ELISpot. Humoral response to KLH was also negative. This patient had a normal total lymphocyte count (except for low CD4+ T- and borderline CD19+ B-cells) both at screening and throughout most of the vaccination calendar yet both humoral and cellular idiotype-specific immune responses were detected, however, only by one of the two analytical methods employed.

(2) Intermediate Responders. About one-half of subjects (6 of 11) in this study exhibited mixed responses to vaccination. For example, patients T006, T010, and U017 uniformly showed negative idiotype-specific humoral responses. Patients T006 and U017 received all 12 vaccines in the series, whereas patient T010 received 9 vaccinations before progressive disease caused removal from study. Patients T006 and U017 showed positive humoral responses to KLH, while T010's KLH response was negative. However, all three patients showed strong idiotype-specific cellular responses as assessed by T-cell proliferation as well as by confirmatory ELISpot. Patient U017's malignant tissue displayed both IgM-kappa and IgG-kappa isotypes and consequently two vaccines were produced. All three patients had normal total lymphocyte counts (except for low CD4+ T cells) both at screening and throughout most of the vaccination calendar.

Patients U016, T022, and U026 received a full course of 12 vaccines; none displayed a positive idiotype-specific humoral response to either the plant-made or hybridoma-made idiotype, but all showed a positive idiotype-specific cellular response as assessed by T-cell proliferation; responses were negative by ELISpot. Only sera from patients U016 and U026 had a positive response to KLH; T022's response was negative. Patients T022 had a normal total lymphocyte count but low CD4+ T- and CD19+ B-cells, while patients U016 and U026 were considerably lymphopenic (all T-cell subsets) throughout the calendar.

(3) Nonresponders. A third group of subjects (2 of 11) comprised patients who did not respond to vaccination. Patient T003 had no measurable humoral or cellular idiotype-specific response throughout the vaccination calendar, in spite of having received the full course of 12 vaccines. Similarly, T008 showed no idiotype-specific humoral or cellular response after receiving 7 of 12 vaccines; the patient progressed after vaccination 4 but went on to receive 7 vaccinations before being removed from study and offered additional systemic

therapy. Both patients did exhibit a humoral response to KLH albeit with a substantial delay. T003 was considerably lymphopenic throughout the calendar. Patient T008 was lymphopenic (except for the number of CD8+ T cells) with a particular depletion of helper T cells. No vaccine-induced immune response was ever detected and this fact seems consistent with a shortage of both B and T cells.

3.4.3. Lack of Immune Responses against Plant Glycans. All idiotypic antigens contained plant-type glycans, including vacuolar type and secretory type complex structures. The reactivity of each patient's immune serum against plant-type glycans was determined by ELISA to further assess potential safety issues borne from plant-specific glycosylation of the antigens. This was seen as particularly poignant in a clinical study of vaccines comprising an immunogenic carrier protein (KLH) and coadministered with a potent cytokine adjuvant (GM-CSF), where immune reactivity against the complex is the desired goal of the treatment.

Table 5 shows the results of this analysis. In each case, antigen coating of the ELISA plates was confirmed by the control antisera. These assays were also used to confirm the absence of nonspecific cross-reactivity from the detecting antisera (see Section 2.12 for detail). Pre- and post-immune sera were analysed for the 11 evaluable patients on study. No evidence for an antibody response to plant glycans, either on the idiotypic vaccine or on horseradish peroxidase, was found in any patient. Some patients had preexisting low titres of glycan-binding antibodies, but the titre was unchanged between pre- and post-immunization sera.

4. Conclusions

We can conclude that the current tumor-targeted, plant-made vaccines manufactured with magnICON technology and administered in combination with the adjuvant GM-CSF are safe when given to adults with relapsed or transformed follicular lymphoma and that the vaccines induce tumor idiotype-specific immune responses. The percentage of patients who successfully mounted a documented vaccine-induced, idiotype-specific immune response (82% positive humoral and/or cellular) compares very favorably with the results of previously published clinical trials with FL idiotype vaccines produced through other manufacturing platforms (e.g., 80% immune response rate, hybridoma-produced, Inogés et al. 2006 [8]; 70% response, plant-produced scFv, McCormick et al. 2008 [12]; 70% response, hybridoma-produced, Inogés et al. 2009 [29]), and is in the range of other FL vaccines administered after non-rituximab-based chemotherapy, as reviewed by Park and Neelapu [30]. In our study, a successful immunologic endpoint was defined as (a) greater than 40% of subjects developing humoral immune responses after receipt of vaccine 6, or (b) greater than 50% of subjects developing cellular immune responses after receipt of vaccine 6. By these criteria, humoral responses were lower than expected but cellular responses exceeded expectations.

The overall immunogenicity to our current vaccine plus adjuvant combination might have been even higher had the patients been monitored for immune responsiveness (immune recovery after chemoreductive therapy) prior to initiation of the vaccination regimen. In relation to a similar study [8] in which 20% (5 of 25) of patients failed to respond to the administered vaccine, in the current study a comparable 18% (2 of 11) evaluated patients seemed to similarly fail, or at least fail to demonstrate, a vaccine-induced, idiotype-specific immune response as assessed by the complementary and independent functional assays performed. One explanation for why we may not have observed a higher rate of double positive responses (i.e., humoral + cellular) is that, in the cited study [8], which featured a hybridoma-produced idiotype, no patient in complete remission (CR) was vaccinated until evidence of B- and T-cell recovery had been demonstrated. In contrast, in the protocol of the current study, the recovery period was fixed at 4 months for practical reasons. In reality, most patients initiated vaccination between 4 and 5 months after conclusion of chemotherapy. Nevertheless, this is shorter than the recovery period adopted in prior studies (e.g., Inogés et al. 2006 [8]; McCormick et al. 2008 [12]; the latter with a 6-month minimum), and it could be expected that the current study had a greater proportion of patients who were not yet immunologically competent (due to insufficient recovery period) at the time of vaccination. These observations are semiquantitative because of the relatively small cohort sizes in all these studies.

It is noteworthy that most patients who failed to mount an immune response by the sixth vaccination were also unable to mount an immune response during subsequent vaccinations. The one exception was patient U017, who was negative for both humoral and cellular responses during most of the calendar until cellular responses were verified during administration of the last vaccines in the series. This observation suggests that a patient's immune status at the beginning of the vaccination calendar may be crucial to the potential development of a vaccine-induced immune response. An ad hoc analysis of minimal residual disease (MRD) versus treatment, as a secondary indicator of efficacy, was added late in the study but yielded no conclusive trends due to the small sample size available for evaluation (data not shown). There is likely room for improving the immunological efficacy of the vaccine via a modified composition, a new adjuvant, or both, plus improving the scheduling for administration to coincide with immune responsiveness. By adopting such changes the clinical benefit of this targeted therapy could be demonstrated in future studies.

In addition to immune responses to the vaccines, this safety study evaluated immune responses to plant-type glycans in the vaccine compositions. Prior studies on plant glycoforms had suggested that certain characteristic linkages in plant-derived complex glycans, particularly β(1,2)xylose and α(1,3)fucose, might be allergenic in humans [31–34]. In our study, a complete analysis of glycosylation was conducted for every plant-manufactured Ig. Extensive heterogeneity was found in the glycan content of the Ig of different patient samples, including oligomannosidic and plant-type complex glycans as well as truncations and mixtures thereof (data not shown). Analyses were also conducted to determine if there was any correlation of glycan content and/or pattern

Table 5: Lack of immune reactivity of patient sera to plant glycans in idiotypic vaccines.

Patient UPIN[1]	Predominant plant glycan type[2]	Vaccinated patient sera		Control[4] glycosylated antibody pre	Control[4] glycosylated antibody post
		Anti-HRP[3] Pre-immune	Anti-HRP[3] Post-immune		
U001	V: GnGnXF S: Gn(FA)XF; (FA)(FA)XF	0.076	0.068	0.071	0.065
T003	V: GnGnXF S: Gn(FA)XF; (FA)(FA)XF	0.081	0.103	0.084	0.080
T006	V: GnGnXF S: Gn(FA)XF	0.106	0.109	0.072	0.068
T008	V: GnGnXF	0.107	0.174	0.100	0.089
T010	V: MGnX S: GnAF/Gn(FA)	0.089	0.105	0.079	0.074
U011	V: GnGnXF S: Gn(FA)XF	0.078	0.069	0.156	0.130
U016	V: GnGnXF S: Gn(FA)XF	0.096	0.082	0.066	0.057
U017G	V: GnGnXF S: Gn(FA)XF	0.094	0.078	0.101	0.064
T021	V: GnGnXF	0.142	0.103	0.072	0.078
T022	V: GnGnXF	0.089	0.136	0.085	0.083
U026	V: GnGnXF S: GnA	0.067	0.069	0.062	0.066
Control anti-plant glycan antiserum (1 : 2000)		0.37		0.515	

1 = UPIN: Unique Patient Identification Number (sera obtained from patients vaccinated as shown in Table 4).
2 = Predominant plant glycan type. V, vacuolar, with major species indicated; S, secretory, with major species indicated. A = galactose; Gn = N-acetyl glucosamine; M = mannose; F = fucose; X = xylose.
3 = HRP, horseradish peroxidase, a plant enzyme containing complex plant-type glycans used as a control.
4 = Irrelevant plant-produced glycosylated human idiotypic Ig used as a control.

in the plant-made vaccines with either clinical safety or immunogenicity (no correlation was found).

As summarized in Table 5, no significant responses were found in any of the patients against the plant glycans associated with any of the idiotype vaccines. In fact, there were no antiglycan responses regardless of whether individuals were strong, intermediate, or weak responders to the vaccines. The results suggest that plant complex glycans are poor or not immunogenic in humans. The prior studies on plant glycan immunogenicity focused on known allergens, and it has been suggested that glycan conformation and the presence of other antigenic determinants may be key to development of immune responses [35]. Our findings are similar to those of McCormick et al. [12], who observed that immunogenicity of plant-produced NHL vaccines was due to the polypeptide component of the idiotype immunogens and that neither safety nor immunogenicity correlated with glycan content or structure. Our results therefore corroborate the growing body of evidence that the plant-type glycan content or structure in plant-produced immunogens is not, *per se*, a safety risk in vaccines administered in low doses by subcutaneous injection, even in the presence of a strong adjuvant.

Our results also validated the significant differentiating features of the magnICON plant-based expression technology used to manufacture the vaccines for this study, including its speed, versatility, and scalability, which are necessary prerequisites for implementation of any customized manufacturing process. The described process is reliable and robust; the total manufacturing time starting from biopsy to a conjugated vaccine is <12 weeks and the expression and purification of antigen required only 2 weeks. The methodology described lends itself to the rapid production of individualized proteins, such as Id NHL vaccine antigens, as well as the prototyping and production of other antigens whose seasonal or mutational variabilities, such as viral pathogens, favor a rapid and flexible manufacturing platform. Together with our earlier findings [18], this process also represents a broadly applicable, robust, scalable, and cost-effective platform for manufacturing monoclonal antibodies in plants, including novel and biosimilar biologics and therapeutic enzymes [36]. Most of the process steps can be automated [37], with the potential application of robotic high-throughput sample processing and cloning adding to the appeal of this flexible platform.

We conclude that customized idiotype vaccines produced by means of the magnICON plant-based expression technology are readily and economically manufacturable and are safe, well tolerated, and immunogenic according to the dose, route of administration, adjuvant, and schedule employed in the current study.

Disclosure

The conduct of the study, data integrity, and reporting accuracy were audited by independent external organizations. Leukine is a registered trademark of Sanofi SA (Paris, France). magnICON is a registered trademark of Icon Genetics GmbH (Halle, Germany). Treanda is a registered trademark of Cephalon Inc. a wholly owned subsidiary of Teva Pharmaceutical Industries Ltd. (Petah Tikva, Israel).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of these results.

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Review Article

Targeted Therapies in Adult B-Cell Malignancies

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B-lymphocytes are programmed for the production of immunoglobulin (Ig) after antigen presentation, in the context of T-lymphocyte control within lymphoid organs. During this differentiation/activation process, B-lymphocytes exhibit different restricted or common surface markers, activation of cellular pathways that regulate cell cycle, metabolism, proteasome activity, and protein synthesis. All molecules involved in these different cellular mechanisms are potent therapeutic targets. Nowadays, due to the progress of the biology, more and more targeted drugs are identified, a situation that is correlated with an extended field of the targeted therapy. The full knowledge of the cellular machinery and cell-cell communication allows making the best choice to treat patients, in the context of personalized medicine. Also, focus should not be restricted to the immediate effects observed as clinical endpoints, that is, response rate, survival markers with conventional statistical methods, but it should consider the prediction of different clinical consequences due to other collateral drug targets, based on new methodologies. This means that new reflection and new bioclinical follow-up have to be monitored, particularly with the new drugs used with success in B-cell malignancies. This review discussed the principal aspects of such evident bioclinical progress.

1. Introduction

B-lymphocytes are programmed for immunoglobulin (Ig) production directed against pathogens via the B-cell receptor (BCR) activation. During this maturation process, B-lymphocytes exhibit different surface markers, activation of intracellular pathways, metabolism modulation, protein synthesis, and interaction with their microenvironment. B-lymphocyte ontogeny takes place in lymphoid organs leading to plasma cells that migrate into the bone marrow or mucosa-associated tissues. Recently, progress in biology knowledge has resulted in a large number of targeted therapies, designed against surface markers, cell signaling pathways, cell cycle and apoptosis machinery, key molecules involved in cellular metabolism, in proteasome, and in immune modulation, and niche disruption. Rituximab, an anti-CD20 monoclonal antibody (mAb), was the first targeted therapy successfully developed in lymphoma and chronic lymphocytic leukemia of B-cell type (B-CLL) [1–3]. A synergy with chemotherapy was demonstrated in all B-cell malignancies (B-CM) expressing CD20 molecules, with significant prolongation of the progression-free survival (PFS) and overall survival (OS) [4]. Beyond rituximab, there are new molecules directed against several factors. This includes (1) other surface markers, including not only other B-cell markers but also receptors (R) of survival/growth factors, such as B-cell activating factor/A proliferation-inducing ligand (BAFF/APRIL)R, interleukin (IL) 6R, IL7R, vascular endothelial growth factor (VEGF)R, epithelial growth factor (EGF)R, stromal cell-derived factor- (SDF-)1R or chemokine receptor type 4 (CXCR4), and insulin-like growth factor (IGF)1R; (2) key points for signaling pathways such as inhibitors of Bruton's tyrosine kinase (BTK), phosphoinositide 3-kinase (PI3K), and spleen tyrosine kinase (Syk); (3) inhibitors of cell cycle regulators; (4) proteasome inhibitors and nuclear factor kappa-B (NFκ-B) inhibitors; (5) metabolism inhibitors, such as antilacticodehydrogenase (LDH); (6) immune modulators; and (7) inhibitors of the interaction between tumor cells and its microenvironment. The complexity of these therapeutic options requires new reflection and approach and new drug combinations based on biological data in order to create optimal conditions for such new age in personalized

medicine, including methodologies, follow-up to evaluate quality of life, and safety and tolerability not only just after the administration, but also after a long treatment period due to improved survival [5] (Tables 1, 2, and 3).

2. Cell Surface Markers

2.1. B-Cell Markers. B-lymphocyte differentiation is associated with the expression of a variety of cell surface markers, including CD19, CD20, C22, CD40, surface Ig, and BAFF-R present at the different maturation steps, excluding the end-stage of this B-cell lineage, plasma cells [6–9]. Conversely, plasma cells also share CD19 and other surface markers such as CD38, CD138, CS-1, CD200, CD56, CD45, and other different markers [10] (Figure 1). In addition, all markers are currently used to define normal and malignant plasma cells, thus allowing evaluation of minimal residual disease, and to establish the true complete response (CR) expressed by the return of normal plasma cells inside the bone marrow niche [11]. Targeting surface B-cell markers also leads to cell signaling, as observed with CD19, CD20, CD5, and CD22 that are B-cell receptor (BCR) coreceptors with either stimulatory or inhibitory activities.

Rituximab, the first anti-CD20 mAb used in humans, has been shown clinically beneficial in B-CM, including non-Hodgkin's lymphoma (NHL) and B-CLL [1–3, 12]. This agent was also developed as a maintenance therapy with a significant prolongation of the therapeutic response. However, empirism was associated with the early development of rituximab, and the usual dose of 375 mg/m^2 was not chosen on a biological basis. The choice of the efficient dose based on biological criteria was only made in the context of B-CLL, with the demonstration of biological efficacy through the quantification of the CD20 molecules at the cell surface and the engagement of the Antibody-Dependent Cell Cytotoxicity (ADCC) [13]. Maintenance therapy with rituximab was rather based on commercial reasons even though treatment prolongation for two years was associated with an improved PFS. In fact, treatment prolongation should have been based on the control of residual disease associated with improved survival. The dose was similar to the one used in chemoimmunotherapy, but with different options for the administration schedule due to a lack of biological rationale, except that ADCC with Natural Killer (NK) cell activation may delay the time of the optimal response.

Delayed complications have been neglected in this context. The first observations were made in patients treated for Rheumatoid Arthritis (RA) who experienced reduction of immune response to vaccination [14] and reactivation of viral infections, not only hepatitis also observed in the context of B-CM, but also progressive multifocal leukoencephalopathy (PML), a lethal encephalitis caused by the polyomavirus JC [15]. We recently observed one case of PML with a severe immune defect due to chemoimmunotherapy and autologous transplantation for NHL, 15 years after the initiation of the therapy (personal data). The fact that similar observations were not so frequent was due to three factors: (1) lack of substantial long-term survivors; (2) the patients having RA had a more pronounced immunodepressed status due to the exposition to several immune modulators, such as corticotherapy, methotrexate, or anti-Tumor Necrosis Factor (TNF) for example; and (3) relative limited efficacy of rituximab in depleting memory B-cells and plasma cell compartment within lymphoid organs [16]. This is not probably the end of the story and longer observation period is needed, particularly with improved efficacy and prolonged patient survival due to new efficient molecules, including the more efficient anti-CD20 mAbs and new targeted therapies. The subcutaneous (SC) form of rituximab was developed as equivalent to the IV formulation. However, the lymph node compartment being the target organ after SC administration was not considered. Had this been taken into account, one could predict a better activity and a better clinical use of the drug. The therapeutic strategy should change, and the current long-term maintenance therapy with rituximab should be avoided. In addition, drug agencies have to prolong patient observation beyond therapeutic response and to analyze the immune response with functional markers, for example, after vaccination [17]. Considerable progress was made in understanding the structure and the functions of CD20 molecules and anti-CD20 mAbs. Binding of the mAbs to their target supports three types of action: intracellular signals leading to programmed cell death, binding to C1q molecules inducing complement-mediated cell lysis, and Fc/FcR interaction or antibody-dependent cell cytotoxicity, particularly with NK lymphocytes [7]. Rituximab, Yttrium-90 ibritumomab, iodine-131 tositumomab, and ofatumumab are all anti-CD20 mAbs approved for different indications and countries, while others are used in clinical trials [7]. Yttrium-90 ibritumomab is an effective therapeutic agent for lymphoma, particularly in the treatment consolidation after immunochemotherapy induction as a first-line treatment for large B-cell lymphoma [18].

As CD19 is expressed by the B-cell lineage, from pro/pre-B-cells to plasma cells, anti-CD19 mAbs may represent good candidates for the treatment of B-CM [8]. Blinatumomab is a bispecific T-cell engager that specifically targets CD19 and CD3 antigens. This bispecific mAb was approved in December 2014 for Acute Lymphoblastic Leukemia (ALL) in USA [19]. In addition, CD19 was used as engineered receptors grafted onto immune effector cells, particularly on T-cells, to generate chimeric antigen receptor T-cells (CAR-T) that express a fusion protein comprised of an anti-CD19 mAb with CD28 costimulatory and CD3-ζ chain signaling domain. This novel technology was developed as adoptive transfer of CAR-T for ALL of B-cell type [20].

The success of rituximab has encouraged developers to propose other mAbs targeting different surface B-cell markers, such as anti-CD22 inotuzumab ozogamicin (CMC-544) or epratuzumab, combined with rituximab [21–23], anti-CD37 particularly for B-CLL [9], and anti-CD74 directed against a component of the HLA DR (milatuzumab) [12, 24]. Epratuzumab induces a marked decrease of CD22, CD19, CD21, and CD79b molecules on the B-cell surface and immune modulation on FcγR-expressing monocytes, NK cells, and granulocytes via trogocytosis [25]. Downstream the receptor, immune signaling involves specific tyrosine residues that are phosphorylated upon receptor activation.

Table 1: Clinical trials for B-cell chronic lymphocytic leukemia (B-CLL), lymphoma (NHL = non-Hodgkin's lymphoma), and multiple myeloma, based on research made by using key words for the different diseases, through https://clinicaltrials.gov/, as of March 13, 2015. VEGF: vascular endothelial growth factor; EGFR: epithelial growth factor; IGF-1 R: insulin growth factor receptor type 1; BTK: Bruton's tyrosine kinase; PI3k: phosphoinositide 3-kinase; HDAC: histone deacetylase; CAR-T: chimeric antigen receptor-T lymphocytes; Cdk: cyclin-dependent kinase; DKK: Dickkopf-related protein; ADCC = antibody-dependent cell cytotoxicity; CDC = complement-dependent cytotoxicity; A = direct apoptosis; M = mouse; H = humanized; Ch = chimeric; C = cytotoxicity; Phag. = phagocytosis; Doxo. = doxorubicin; Cytotox. = cytotoxicity.

		Type of mAb biological activity	Number of studies Lymphoma 2246 studies	B-CLL 1965 studies	Multiple myeloma 1908 studies
Monoclonal antibodies	Anti-CD19		65	34	3
	Blinatumomab CD19/CD3				
	Anti-CD20		1017	329	20
	Rituximab (CLL, NHL)	Ch IgG1 ADCC, CDC, A			
	Ofatumumab (CLL)	HIgG1 CDC+	58	57	—
	Obinutuzumab	ADCC+	19	26	—
	Ocrelizumab	ADCC+	3	1	—
	Veltuzumab	ADCC, CDC	7	1	—
	Ibritumomab tiuxetan (NHL)	MIgG1			
	Tositumomab (naked or ^{131}I)	HIgG1	40	5	
	NHL				
	Anti-CD22		16	21	—
	Epratuzumab (naked or ^{90}Y)	HIgG1 trogocytosis			
	Epratuzumab immunotoxin				
	Anti-CD25, *LMB-2*	Anti-TAC(Fv)PE38, C	2	—	—
	Anti-CD38 daratumumab	HIgG1, ADCC, CDC, A	—	—	6
	Anti-CD40		11	19	20
	Dacetuzumab	HIgG1, ADCC, phag.			
	Anti-TRAIL 2*conatumumab*	Agonist HIgG1, A			
	Anti-CD45		7	9	5
	BC8 ^{131}I/BC8 ^{90}Y	MIgG1			
	Anti-CD74 hLL1 *milatuzumab*		1	4	2
	(+doxo)	HigG1, A/Cytotox.			
	Anti-CD80 galiximab	HIgG1, ADCC	4	1	—
	Anti-CTLA4 ipilimumab	HIgG1, ADCC	7	14	3
	Anti-PD-1 nivolumab	HIgG4	2	9	1
	Pidilizumab	HIgG1	—	1	1
	Anti-VEGF (sorafenib,		21	6	13
	bevacizumab)	CIgG1			
	Anti-IGF-1R	HIgG1	1	1	3
	Anti-IL6, *siltuximab,* and	CIgG1			7
	tocilizumab	HIgG1	—	—	2

Table 1: Continued.

		Type of mAb biological activity	Number of studies Lymphoma 2246 studies	B-CLL 1965 studies	Multiple myeloma 1908 studies
BTK inhibitor	*Ibrutinib* *spebrutinib,* and*ONO-4059*		9	47	3
PI3 kinase/Akt/mTOR/PIM/MEK inhibitor	Idelalisib, duvelisib, and TGR-1202 RP6530 (dual PI3K$\delta\gamma$) MK2206, AMG 319 LGH447, BYL719 Pictilisib (GDC-0941) GSK1120212, GSK110183 Nelfinavir, CUCD-907		23	14	8
Proteasome inhibitor	Ixazomib, salinosporamide, Filanesib, oprozomib, and lapatinib		20	6	77
HDAC	Vorinostat, ricolinostat, panobinostat, givinostat, 4SC202, entinostat, quisinostat, rocilinostat, tacedinaline, abexinostat, and CDX101		135	26	56
CAR-T	CD19, CD30, CD20, CD22, and CD138		13	24	3
Other drugs	Somatostatin analog (pasireotide) Anti-DKK1 CDK inhibitors Anti-EGFR (erlotinib, crizotinib)		13 2	6 —	1 1 5 —

Table 2: Clinical trials for lymphoma: (a) for follicular lymphoma; (b) for Mantle cell lymphoma, based on https://clinicaltrials.gov/, as of March 13, 2015. PD-L-1: programmed death-1 ligand 1; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; HDAC: histone deacetylase; PI3k: phosphoinositide 3-kinase; BTK: Bruton's tyrosine kinase; Cdk: cyclin-dependent kinase; bcl2: B-cell lymphoma 2; PARP: poly(ADP-ribose) polymerase; ASCT: autologous stem cell transplantation.

(a)

Follicular lymphoma 1031 studies	Monoclonal antibodies 232 studies	Anti-PD-1	2 studies	Combination with rituximab
		CD20 radio immunotherapy	34 studies including Phase 3	Combination + ASCT
		CD45 ^{131}I	1 study Phase 2	+ ASCT
		Anti-CTLA-4	1 study	Combination with SD-101 (TLR9 agonist)
		Anti-CD20	10 studies Phase 1 Phase 2	+ lenalidomide maintenance
		Bevacizumab	Phase 1 Phase 1	Combination + vandetanib
		Apolizumab (anti-DR)	Phase 1 Phase 2	
		Galiximab (anti-CD80)	Phase 2 1 study	+ rituximab
		Anti-CD19	Phase 1 2 studies	
		Anti-CD22	Phase 1/2	+ ASCT
		Radioimmunotherapy	Phase 2/3	
		Cold	Phase 1	
		Anti-CD74	Phase 1/2	Combination with rituximab
		Anti-CD20+IL12		
		Anti-$\alpha v\beta 3$ integrin	Phase 1	
		Anti-CD80	Phase 1/2	
		BMK120	Phase 1	Rituximab
	PI3K inhibitor	Buparlisib		
		BAY80-6946	Phase 2	
		Idelalisib	Phase 3	Combination
		Entospletinib		Phase 1
	BTK inhibitor	Ibrutinib/ONO 4059	Phase 2	10 studies
		Spebrutinib		
	Anti-CDK	Flavopiridol	Phase 1	Combination
	Anti-Bcl-2	Antisense	Phase 2	Combination with rituximab
			2 studies	
		Obatoclax	Phase 1/2	
	Anti-PARP	Alisertib	Phase 2	Combination
		Veliparib	Phase 1/2	
	HDAC	Vorinostat	Phase 2	+ rituximab
	Anti-kinase	Vandetanib	Phase 1	

(b)

		Anti-CD20		
		Rituximab	Phase 2 Phase 3	Chemo, vorinostat, bortezomib
		Ofatumumab	Phase 1	

(b) Continued.

		Ublituximab ^{90}Y/^{131}I		+ lenalidomide maintenance + ASCT
		51 studies Anti-CD56 ^{131}I 3 studies	Phase 1	+ ASCT
	Monoclonal antibodies 158 studies	Anti-VEGF bevacizumab	Phase 2	3 studies
		Anti-VEGF kinase (cediranib)	Phase 1	+ bevacizumab
		Anti-transferrin R	Phase 1	
		Anti-CTLA4	Phase 1/2	4 studies
		Anti-HLA DR	2 studies	Phase 1
		Anti-CD22	Phase 1	1 study
		Anti-CD22 ^{90}Y	Phase 1/2	+ anti-CEA In111 1 study
Mantle cell lymphoma 860 studies		Anti-CD22 In111	Phase 1/2	
		Anti-α-v β3 integrin	Phase 1	
		Anti-CD19	Phase 1/2	
		Anti-CD74	Phase 1/2	+ veltuzumab (humanized MoAb)
		Anti-IGF-1R ganitumab	Phase 1/2	
		Anti-TRAIL R2 conatumumab	Phase 1	+ bortezomib/vorinostat
	Anti-PI3K	Idelalisib	Phase 1	Chemo/rituximab
		BKM120	Phase 1	+ rituximab
	Anti-BTK	Ibrutinib	Phase 1	Chemo/rituximab
	Anti-cdk	Flavopiridol	Phase 1	+ chemo/rituximab
	mTOR inhibitor	Temsirolimus	Phase 2	+ rituximab
			Phase 1/2	+ cladribine/rituximab
	Anti-endosialin/TEM1		Phase 1	
	HDAC	Romidepsin	Phase 1/2	+ rituximab/lenalidomide
	Anti-bcl2	Oblimersen	Phase 2	+ rituximab
		Obatoclax		+ chemo/rituximab
	Aurora-kinase inhibitor	Alisertib	Phase 2	+/− rituximab
	Dehydrogenase inhibitor	CPI-613	Phase 1	+ bendamustine/rituximab
	HDAC	Vorinostat	Phase 1/2	+ chemo
	Toll-R agonists	CPG 7909	Phase 2	+ chemo

These phosphorylation sites are frequently found in one of the three types of Immunoreceptor tyrosine-based regulatory motifs (ITRM), including IT activation M (ITAM), IT inhibition M (ITIM), and IT switch M (ITSM) for SLAM/CD150 and related receptors of the CD2 subfamily [26]. Generally, ITIMs recruit the SH2 domain-containing tyrosine phosphatase SHP-1 or SHP-2, and phosphorylated ITAMs are recognized by SYK in B-cells [27, 28].

Epratuzumab combined with rituximab was associated with a high response rate including 42.4% of CR rate with 60% of the patients having 3-year remission, for untreated patients with follicular lymphoma (FL) [21]. This relatively high response rate is not superior to that observed with other treatments, but it opens the pathway for targeted therapy without chemotherapy. However, the combination of two mAbs is less cost-effective compared to new targeted drugs used orally; a decision was made to discontinue its development. A possible way for such development would be radioimmunotherapy and utilizing Yttrium-90 epratuzumab or other combination of CD22 with calicheamicin, or with PE38, a fragment of *Pseudomonas* exotoxin or novel anti-CD22 mAb that blocks CD22 ligand binding, or second generation ADCC with linkers and more potent toxins, particularly tried in ALL [22, 23].

CD19, CD200, CD38, CD138, CD56, and CS-1 are major targets expressed on Multiple Myeloma (MM) cells. MAbs

Table 3: Clinical trials for multiple myeloma, based on https://clinicaltrials.gov/, as of March 13, 2015. ASCT: autologous stem cell transplantation; PD-L-1: programmed death-1 ligand 1; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; IGF-1R: insulin growth factor-1 receptor; KIR: killer cell Ig-like receptor; DKK: Dickkopf-related protein; BTK: Bruton's tyrosine kinase; PI3k: phosphoinositide 3-kinase; PARP: poly(ADP-ribose) polymerase.

Multiple myeloma 1908 studies	Monoclonal antibodies 82 studies	Anti-CD38	4 studies	Phase 2
		Anti-IL6 siltuximab	5 studies	Combination
		Anti-CD40	4 studies	Phase 1/2
		Anti-transferrin R	1 study	Phase 1/2
		Anti-GM2	1 study	Phase 1/2
		Anti-CD66 ^{90}Y	1 study	+ ASCT
		Anti-CD45 ^{90}Y	1 study	+ allogeneic transplantation
		Anti-Adhesion Mol1	1 study	Phase 1
		Anti-CD38	1 study	Combination
		Anti-PDL1	3 studies	Phase 2 with vaccination
				Combination with lenalidomide
		Anti-IGF1R	1 study	Phase 1
		Bevacizumab	3 studies	Phase 2 combination
		Anti-KIR	4 studies	Phase 1 and Phase 2
		Anti-CTLA-4	2 studies	+ allogeneic T.
		Anti-CD52	3 studies	+ allogeneic T.
		Anti-DKK1	1 study	Randomized Phase 2
		Anti-CD20	^{90}Y/^{131}I 2 studies	+ ASCT
			Cold: 3 studies	Combination
		Anti-CD56	1 study	Phase I
		Elotuzumab	5 studies	Randomized Phase 1/2
		Anti-GRP78 (PAT-SM6)	1 study	Phase 1
		Anti-CXCR4	1 study	Phase 1b
	BTK inhibitors	Ibrutinib	2 studies (+ carfilzomib)	Phase 2
		ACP-196/ACP-319	3 studies	Phase 1b
	PI3 kinase inhibitors	Idelalisib, BYL719, CUDC-907, nelfinavir, SOM230 LAR, and sorafenib		Phase 1/2
	Anti-CDK	Dinaciclib	Combination	Phase 1/2
	Antiproteasome	Carfilzomib	10 studies	Phase 3
		Ixazomib		
		Oprozomib		
		Marizomib		Phase 1
	HDAC	Ricolinostat, vorinostat	Combination	Phase 1b
	PARP inhibitor	ABT-888	Combination	Phase 1

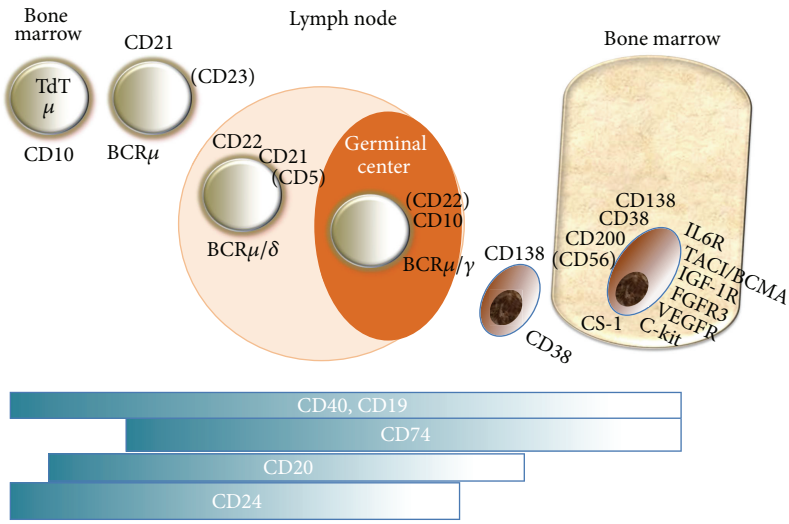

Figure 1: Surface markers of B-cell lineage present at the principal stages of differentiation, as targets for therapy. TdT: terminal deoxynucleotide transferase. TACI/BCMA: transmembrane activator and CAML interactor/B-cell maturation antigen. IGF-1R: insulin growth factor-1 receptor. EGFR: epithelial growth factor receptor. VEGFR: vascular endothelial growth factor receptor. IL6R: interleukin 6 receptor. FGFR3: fibroblast growth factor receptor type 3. c-Kit: CD117. BCR: B-cell receptor.

against such molecules have been clinically developed [29]. Elotuzumab, a humanized mAb IgG1 antibody that targets CS-1 (SLAMF7), a cell surface glycoprotein with major expression in MM cells, has been shown to support very active ADCC [30]. It has been combined with lenalidomide and dexamethasone in patients having relapsed MM with promising results, 90% of the patients achieving a partial response (PR) with PFS exceeding 2 years [30]. A Phase III clinical study is ongoing and is due to be completed by 2017. Daratumumab is a humanized antibody against CD38 [31], a cell surface protein strongly expressed in MM [32]. CD38 is also expressed on malignant cells from B-CLL, mantle cell lymphoma (MCL), transformed FL, and clinical trials are ongoing with daratumumab in these diseases [31]. Some MM cells expressed CD56 and lorvotuzumab, an mAb against CD56, conjugated to mertansine has been developed in early clinical studies for MM [33]. CD200 is an immunosuppressive molecule overexpressed in several hematological malignancies including B-CLL, MM, and acute myeloid leukemia (AML) [34, 35]. Early clinical trials are ongoing in these diseases or in different models of immunotherapeutic strategies in AML [35]. Syndecan-1 (CD138) belongs to heparan sulfate proteoglycans that is highly expressed at the cell surface of MM cells [32, 36]. In addition, cell surface CD138 acts as a functional coreceptor for chemokines and growth factors in the plasma cell niche. Soluble form of syndecan-1 can accumulate survival factors within the microenvironment, representing a sort of sponge for these factors around the tumor cells [36]. Therefore, targeting this molecule is of potential clinical interest, due to a mixed activity on both the tumor cells and its cell niche, making the molecule attractive for radioimmunotherapy [37]. Different mAbs have been developed in early clinical phases including anti-CD40 mAbs such as lucatumumab, dacetuzumab, or mAb directed against HM1.24, the XmAb 5592 [38]. A total of 91 studies with mAbs are registered (https://clinicaltrials.gov/ct2/results?term=monoclonal+antibodies+in+multiple+myeloma&Search=Search) in MM patients, as of March 13, 2015.

For all of these mAbs and similarly to rituximab, clinical efficacy was only observed with combination strategies, particularly with other active drugs, depending on the response or the refractoriness to prior therapies, including bortezomib, IMiDs such as lenalidomide plus dexamethasone, and other new active drugs including approved molecules such as pomalidomide and carfilzomib, or other new targeted molecules.

2.2. Survival/Proliferation Factor Receptors. Upon recognition of foreign antigens, mature naive B lymphocytes are activated, leading to the production of short-lived plasma cells, followed by their proliferation and differentiation into memory B-lymphocytes and long-lived plasma cells for durable Ig production [39, 40]. Along these different steps, B-lymphocytes respond to diverse signals or survival/proliferation factors, including BAFF/APRIL, BCR, IL6, VEGF, EGF, and IGF-1 [39]. By blocking the specific receptor or neutralizing the ligand, the activation of signaling pathway is not delivered into the cell, leading to tumor cell growth and/or survival arrest. BAFF and APRIL belong to the TNF family that binds to the TNFR-like receptors transmembrane activator, particularly interacting with three receptors, calcium modulator and cyclophilin ligand interactor (TACI), B-cell maturation antigen (BCMA), and BAFFR for only BAFF [41, 42]. APRIL is produced by hematopoietic cells, particularly by osteoclasts [43]. The inhibition of BAFF and APRIL using a soluble receptor, TACI-Ig or atacicept (SeronoMerck Inc.), in a culture of myeloma cell lines causes rapid cell death [44] and inhibits myeloma growth in a coculture system with osteoclasts [45]. We used this drug in a Phase I study, in patients with MM and macroglobulinemia,

with promising results, but this drug was mainly developed in dysimmune diseases [46–48].

Different mAbs against IL6 or soluble IL6R have been developed, particularly siltuximab, an anti-IL6 mAb, and tocilizumab, an antisoluble IL6R. Siltuximab has been recently registered for Castleman's disease in Europe and USA. Tocilizumab is registered for some dysimmune diseases refractory to anti-TNF worldwide and Castleman's diseases only in Japan [49].

IGF-1 represents the main cell communication factor produced by plasma cells and bone marrow stromal cells [50]. Inhibitors of IGF-1, including dalotuzumab and picropodophyllin, have been tested in cancers including early clinical phases of MM [51, 52]. However, as observed for IL6, the use of such specific inhibitors in very advanced diseases did not show any clinical benefit due to intraclonal heterogeneity, with the emergence of tumor cell independence from their microenvironment in addition to other growth factors [53].

3. Intracellular Targets

3.1. Cell Signaling Markers. The activation of the BCR is a major signaling pathway for B-lymphocyte function. The BCR is a multiprotein structure with a surface transmembrane Ig noncovalently associated with the Igα (CD79A) and Igβ (CD79B) chains [54, 55]. Antigen binding to the BCR causes receptor aggregation and engagement of the signal transduction via the phosphorylation of the receptor's cytoplasmic tyrosine-based activation motifs (ITAMs) by recruited SRC-family kinases, including LYN, FYN, BLK, and LCK [54]. Then, the activation of phosphoinositide 3-kinase (PI3K3δ) mediates the conversion of phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-trisphosphate and ultimately recruits BTK [56]. BTK phosphorylation targets phospholipase Cγ2 (PLCγ2), with activation of NFκB and mitogen-activated protein kinase pathways. Antigen-independent signaling has been involved in B-CM which results in constitutive or aberrant BCR signaling, making BTK a major target for such diseases [57]. Ibrutinib (PCI-32765, Imbruvica, J&J Inc.) has been developed in B-CLL and B-cell lymphoma and is now approved for MCL and B-CLL. In a Phase II clinical study, a dramatic response rate was observed in both diseases, particularly in MCL with refractory disease (objective response rate (ORR) 68% including 21% CR) with a median PFS of 13.9 months [55]. Ibrutinib inhibits the adhesion mediated by chemokine and integrin to their microenvironment. This biological effect is associated with lymphocytosis and nodal shrinkage. This lymphocytosis decreased generally at the end of cycle 2. Tolerability was acceptable and adverse events included diarrhea (50%), fatigue (44%), nausea (38%), cough (31%), and myalgia (25%). As ibrutinib is metabolized by cytochrome P450 enzyme 3A (CYP3A), coadministration with CYP3A inhibitors or inducers can interfere with other drugs and may be responsible for some toxicity. Ibrutinib was associated with chemotherapy, bendamustine, or the CHOP-R regimen that associates cyclophosphamide, doxorubicin, vincristine, prednisone, and anti-CD20 mAbs, particularly in B-cell lymphoma [55]. Currently, ibrutinib is used in naive patients, especially for B-CLL and small lymphocytic lymphoma. It showed a high response rate of 71% including 13% CR, with estimated PFS and OS at 2 years of 96.3% and 96.6%, respectively, at the daily dose of 420 mg [58]. In MM, the overexpression of BTK varied, being more present in MM than in monoclonal gammopathy of undetermined significance (MGUS), with also some interindividual variability of the expression level. Despite this variability, ibrutinib was associated with a high response rate in patients with refractory MM [59].

Ibrutinib has promising activity in other B-CM, including atypical B-cell lymphoproliferative disorders (personal data) and dysimmune diseases. The active dose could be correlated with the expression level of the targeted molecule, and its measurement could be a guide to optimize the clinical efficacy. In addition, since this drug is also active in patients with poor prognostic factors such as p53 mutation or other acquired genetic modifications, there is a need to define new markers of interest and new therapeutic combinations including immune therapy to prolong the therapeutic response. Knowing the mechanisms of resistance, the effect on the normal B-cell compartment and other immune cells, the status of the immune response and following the residual disease may contribute to addressing the question of the optimal treatment duration, to avoid the mistakes made with IMiDs in MM [60]. Some resistance mechanisms have been studied, including NFκB pathway and KRAS mutations. The effects of ibrutinib on normal immune cells begin to be studied, including IL2-inducible kinase that promotes a T helper 1 response, a depletion of the B-cell memory and long-lived plasma cell compartment, thus reducing a recall response or a new antigen-dependent response.

Several other BTK inhibitors are in clinical development, including ONO-4059 (Gilead Inc., USA) and AVL-292 (Celgene Inc., USA) which are reversible inhibitors of BTK. In addition, there are multikinase inhibitors, such as LFM-A13, which inhibits BTK and polo-like kinase (PLK), fostamatinib, which inhibits the δ isoform of PI3K and Syk [61], and dasatinib, initially developed as an inhibitor of tyrosine kinase for CML patients, which is also a BTK inhibitor.

The PI3 kinase (PIK)/AKT/mTOR pathway is an important signalling pathway for cellular functions, particularly growth and metabolism control. Different classes and isoforms of PI3Ks exist that are associated with large possibilities of inhibition leading to a great number of molecules inhibiting this pathway. IPI-145 inhibits PI3K δ and γ, and it was developed in hematological malignancies. BAY 80-6946 predominantly inhibits PI2Kα, δ isoforms, as well as INK1117, a PI3Kα inhibitor, and more than 30 other compounds. Among them, idelalisib (GS-1101, Zydelig, Gilead Inc.), a specific inhibitor of class I isoform p110δ was approved on July 23, 2014, in USA for the treatment of FL and B-CLL and B-cell small lymphocytic lymphoma [62]. This molecule is also active in other B-CM.

The combination of these kinases inhibitors with mAbs requires the evaluation of the impact of such molecules on effector cells, particularly NK lymphocytes. Ibrutinib did not inhibit complement activation or complement-mediated lysis. In contrast, ibrutinib and idelalisib strongly inhibited cell-mediated mechanisms induced by anti-CD20 mAbs,

particularly the activation of NK lymphocytes [63]. In addition, idelalisib reduces T-regulator lymphocytes (T-regs) and could have a positive impact on tumor cells [64].

3.2. Cell Cycle, Proteasome, and Apoptosis Machinery. In cancer, proliferation depends on different proteins involved in cell-cycle regulation, particularly alterations of the cyclin-dependent kinase (CDK) CDK4/6-INK4-Rb-E2F cascade [65]. Resistance to chemotherapy is mediated by dysregulation of the cell-cycle machinery [66]. Overexpression of cyclins (e.g., cyclins D1 and E1), amplification of CDKs (e.g., CDK4/6), inactivation of critical CDK by CDK inhibitors (I) (e.g., $p16^{Ink4a}$, $p15^{Ink4b}$, $p21^{waf1}$, and $p27^{kip1}$), loss of retinoblastoma (Rb) expression, and loss of binding of CDKIs to CDKs (e.g., INK4 binding to CDKs) occur frequently in human malignancies [65]. Defects of apoptotic pathways are often observed in hematologic malignancies, involving the global repression of transcription by drugs that inhibit CDK7/9 [67]. Transcriptional CDKIs downregulate a great number of short-lived antiapoptotic proteins. This includes the antiapoptotic proteins myeloid cell leukemia-1 (Mcl-1) particularly critical in hematologic malignancies, the B-cell lymphoma extra long (Bcl-xL) and the XIAP (X linked IAP), D-cyclins, c-myc, Mdm-2 (leading to p53 stabilization), p21waf1, proteins whose transcription is mediated by nuclear factor-kappa B (NF-κB), and hypoxia-induced VEGF [68].

Molecules that interfere with CDKs have been developed, either targeting a broad spectrum of CDKs or a specific type of CDK or targeting CDKs as well as additional kinases such as VEGFR or platelet-derived growth factor-R (PDGFR). More than 10 molecules have gone through clinical trials, including multi-CDK inhibitors such as flavopiridol (Sanofi-Aventis Inc.), a semisynthetic flavonoid, known as a pan-CDK inhibitor, developed in a large panel of hematological malignancies, SNS-032 (BMS-387032, Sunesis, BMS Inc.) developed in B-CLL, MM, and NHL, dinaciclib (SCH 727965, Merck Inc.) and PD0332991 (Pfizer Inc.) developed in various hematological malignancies, and R-roscovitine (seliciclib, CYC202, Cyclacel Inc.) [69]. The combinations of such inhibitors with cytotoxic agents but also with novel and targeted agents, including histone deacetylase inhibitors and PKC activators, NFκB pathway modulators, and probably BTK and PI3K inhibitors, are programmed for clinical trials.

The ubiquitin proteasome pathway plays a critical role in regulating many processes in the cell, which are important for tumor cell growth and survival. Bortezomib was the first clinical success in some cancers and has prompted the development of the second generation of proteasome inhibitors. The ubiquitin proteasome system represents the major pathway for intracellular protein degradation, with a complex mechanism involving at least six components: ubiquitin (Ub), the Ub-activating (E1), a group of Ub-conjugating enzymes (E2), a group of Ub ligases (E3), the proteasome, and the deubiquitinases, a process that is highly controlled in normal cells, but frequently dysregulated in cancers [70].

Chemotherapy designed cytotoxic drugs which are active through impairing mitosis or fast-dividing cells by various mechanisms including damaging DNA and inhibition of the cellular machinery involved in cell division. The number of dividing cells is estimated by the mitotic index, the presence of Ki-67 positive cells on tumor samples, or the percentage of cell cycling in S phase. Such analysis may guide the prescription of cytotoxic drugs, particularly for cancers with variable percentage of cycling cells like in MM with high proliferative index superior to 4% of cells in S phase [71]. The inhibition of NFκB activity modified the degradation of cell cycle-related molecules and perturbed proapoptotic and antiapoptotic protein balance, endoplasmic reticulum stress and inhibited angiogenesis and DNA repair, all the mechanisms that contribute to apoptosis of tumor cells. NFκB that is constitutively active in a large proportion of cancers and is bound to its inhibitor IκB within the cytoplasm, and inhibition of proteasome activity prevents degradation of IκB and subsequent activation and translocation of NFκB to the nucleus. Proteasome inhibitors may induce cell cycle arrest by interfering with the degradation of cell cycle regulators including cyclins. There are several inhibitors of proteasome that are used in clinic for hematological malignancies, particularly for MM and MCL, and used in combination with different other drugs such as IMiDs, other cytotoxic molecules, and dexamethasone. Major proteasome inhibitors include bortezomib, carfilzomib, but also NPI-0052, a β-lactone derived from the marine bacterium *Salinispora tropica*, MLN9708, CEP-18770, ONX0912, or inhibitor of the immunoproteasome such as ISPI-101 or PR-924 [70].

Apoptosis is a common process of cell death for all multicellular eukaryotic organisms leading to the eradication of damaged or infected cells. Apoptosis is initiated by two signaling pathways, the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway, that is, Fas/CD95 that binds to specific cell surface receptors. The intrinsic pathway, with members of BCL2 family, is more commonly perturbed in lymphoid malignancies, including mutation of the tumor suppressor gene TP53, which normally acts to activate certain BH3-only proteins, and the overexpression of BCL2 [72].

Obatoclax (GX15-070) is a pan-BCL2 family inhibitor, binding to BCL2, $BCLx_L$, BCLw, and MCL1. Therapeutic response with obatoclax in clinical trials has been reported to be low and its development has been halted [68]. The natural product gossypol and its synthetic derivative AT-101 bind to BCL2, $BCLx_L$, and MCL1 with clinical activity only when combined to rituximab for FL [73]. Antiapoptotic BCL2 proteins antagonize death signaling by heterodimer formation through binding at the BH3 domain of the protein. New molecules, BH3 mimetics were designed to functionally antagonize BCL2 protein family [74]. ABT-737 and its orally available analogue ABT-263 (navitoclax) bind and inhibit BCL2, $BCLx_L$, and BCLw with high affinity, and it is developed in clinical phases, as well as ABT-199 which may be considered as the most active drug in the BCL2 family inhibitors. ABT-199 has shown high response rate (87%) in relapsed/refractory B-CLL, including bulky disease, fludarabine-refractory disease, and del17p patients [75], as well as for FL, Waldenström's disease, and MCL [73]. ABT-199 induces apoptosis within 8 hours and the most significant dose-limiting toxicity is tumor lysis syndrome. In addition, ABT-199 may be combined to chemotherapy, demethylating

agents, histone deacetylase inhibitors, and novel targeted drugs such as ibrutinib and idelalisib [76].

3.3. Metabolic Process. In the early twentieth century, Warburg first discovered that cancer cells preferentially consume glucose and metabolize it to lactate in the presence of oxygen, named aerobic glycolysis [77]. Accumulated evidence was made to support that this metabolic way was predominant for hematological malignancies in leukemias and T-cell lymphoma, with both inducers of Warburg effect, PKM2, and HIF-1α, reported to be involved in AML and connected to epigenetic control of gene expression [78, 79]. This metabolic process facilitates cancer progression by resisting induction of apoptosis and promoting tumor metastasis or independence of the cancer cell microenvironment. Hypoxia is a major factor that contributes to the Warburg effect, for rapid energetic production for the cancer cell, a process favored by changes within the microenvironment. Blocking glycolysis causes a rapid dephosphorylation of BAD protein at Ser112, leading to BAX localization to mitochondria and important cell death, also observed in multidrug resistant cells [80]. The uptake of fluorodeoxyglucose positron emission tomography in cancers demonstrates the key role of glucose in the proliferation of cancer cells [81]. The generic drug dichloroacetate is a small orally available molecule known to block the pyruvate dehydrogenase kinase. It has thus been proposed in various cancers including rare patients with hematological malignancies and its use was associated with some success [82]. Through the reduction of SIRT1, the inhibition of LDH-A provides a way of altering p53 acetylation status and the downstream induction of p53 target genes selectively in cancer cells [83]. Other target is represented by peroxisome proliferator-activated receptor (PPAR), a group of nuclear receptor proteins that function as transcription factors regulating gene expression. PPAR-α is particularly implicated in lipid and lipoprotein metabolism and inflammation. Fenofibrate, a PPAR-α agonist, has been shown to induce apoptosis on certain cancer cells via activation of NF-κB pathway [84]. Inhibitors for PPAR-γ may enhance the activity of radiation therapy in cancer [85].

There are several compounds that modulate glycolytic metabolism. This includes 2-deoxyglucose that inhibits phosphorylation of glucose hexokinase (HK), lonidamine, that inhibits glycolysis and mitochondrial respiration, HK, 3-bromopyruvate that inhibits HK and acts as an alkylating agent, imatinib that inhibits bcr-abl tyrosine kinase but also decreases HK and G6PD, and oxythiamine that inhibits pyruvate dehydrogenase [86, 87]. Specific LDH inhibitors have been developed, including AT-101, FX-11, galloflavin, N-hydroxyindole-based molecules [88], or new molecules in development by different companies. Such new molecules represent a new potent way to modulate or prevent chemoresistance. In addition, they may have some impact on immune cells [89].

4. Targeting Microenvironment

4.1. Immune Therapy. The tumoral microenvironment, and particularly immune cells, is involved in the tumor cell control or expansion. Since many years, it has been recognized that T-infiltrating lymphocytes (TIL), a mixture of different cells (Treg, T helper, T cytotoxic cells, etc.) when expanded *ex vivo*, may support some clinical efficacy [90, 91]. Nowadays, the different cell subpopulations associated with a particular function (i.e., facilitating or repressing tumor cells) may orientate the clinical prognosis and the response to therapeutic agents [92–98]. Targeting cancer cells via the immune system depends on the presence of effector cells that recognize and kill cancer cells. Recognition may be specific for adaptative response, that is, cytotoxic T-cells via antigen presentation. In the context of innate response, there are other mechanisms to recognize stress cells or non-self-cells, including activating and inhibiting molecules shared by NK, NKT, and T$\gamma\delta$ lymphocytes [99]. Such cancer cell recognition may be forced by using chimeric antigen cells (CAR-T cells, CAR-NK cells) [20] or bispecific mAbs. Beyond recognition, target accessibility and tumor infiltration, mechanisms, and efficacy of killing are other criteria of efficacy to be considered. Effector cells could be directly used as cell-drugs or immune modulators that activate such specific activity, including Toll-receptor agonists [100], enhancers of ADCC and antigen presentation via dendritic cells [101], and stimulator of T$\gamma\delta$, particularly $\gamma 9\delta 2$ T-cell, that may be purified for cellular therapy programs and activated by IL2 and bisphosphonates or IPH101 ([102–104] and personal data) combined with anti-CD20 mAbs [93]. It is surprising that using GM-CSF in addition to rituximab or IPH101 plus IL2 and rituximab, in relapsed or refractory FL, we observed similar results with 45–50% of ORR ([105] and personal data), meaning that optimal strategy is probably the direct administration of these effector cells. Development of NK cells is now one major way for immune therapy probably by using banked, activated, and amplified NK cells from cord blood samples ([106, 107] and personal data). In that way, it is important to know the efficacy of killing. For NK cells, *in vitro* data showed that one NK cell may kill 8–10 tumoral cells. Conversely, 10 cytotoxic T-cells are needed to kill one tumoral cell. This shows that NK-cell drugs are more efficient for killing, with a clinical efficacy ranging between 10^7 and 10^9 tumoral cells. But cytotoxic T-cells may retain a certain memory of killing and prolong the effect. This means that clinical use of such cell-drugs has different clinical targets and could be associated for a better clinical benefit. We need to simplify the therapeutic strategies and think about best combinations of drugs, cell-drugs, modifiers, and new targeted therapies.

4.2. Niche Disruption. Lymph node microenvironment includes different types of lymphocytes and stromal cells necessary to the antigen presentation and the education of B-cells to secrete specific antibodies. Plasmablasts generated in germinal centers exit the lymphoid organs into the lymph and then the blood, before migrating to the bone marrow or mucosa-associated lymphoid tissues where they represent a long-lived population of plasma cells in a favorable microenvironment, named plasma cell niche. Different cells constitute this niche, particularly mesenchymal cells that produce chemokines, particularly CXCL12, and bring together other niche cells (megacaryocytes, platelets, and

eosinophils) and plasma cells, which all express the CXCL12 receptor, CXCR4 [108]. Within the niche, plasma cells are activated by adhesion molecules and stimulated by several survival/growth factors [109]. The hypoxic microenvironment plays a central role in controlling stem cell phenotype and dissemination, through different factors particularly the hypoxia-inducible factor-1α (HIF-1α), a key transcriptional factor that responds to hypoxic stimuli [110]. HIF-1α is constitutively expressed in some B-cell malignancies and is regulated by the PI3K/AKT pathway [111].

Anti-CXCR4 or CXCL12R (plerixafor and others), anti-CCR5 or CCL5R (maraviroc), inhibitors of survival/proliferation factors, that is, IL6, BAFF/APRIL, and others, but also inhibitors of osteoprotegerin, and a receptor for both RANKL and TNF-related apoptosis-inducing ligand/Apo2 (TRAIL) may represent new targets for cancer therapy [49, 112, 113]. The complex CXCL12/CXCR4 is implicated in biological mechanisms of several B-cell malignancies, particularly for CLL, MM, and lymphoma [112]. Plerixafor/AMD3100 disrupts the B-CLL microenvironment interactions, representing additional treatment, possibly with novel targeted drugs [114].

Syndecan-1 is a member of the heparan sulfate (HS) proteoglycans that are present on the cell surface or as soluble molecules shed from the cell surface. Syndecan-1 accumulates survival factors within the microenvironment, representing a sort of sponge for these factors around the tumor cells. Syndecan-1 is cleaved by heparanase, an endo-β-o-glucuronidase, secreted by osteoclasts [36]. As heparin and low molecular weight heparin have been known since a long time to exhibit potent antiheparanase activity, one can explain that such molecules may have a clinical impact on the cancer [115].

A new therapeutic era is born for new reflection, new methodologies and, nowadays, nearly all therapies are targeted as long as we understand biological processes for a better use of old and new drugs to support personalized medicine.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Research Article

Subcutaneous Administration of Bortezomib in Combination with Thalidomide and Dexamethasone for Treatment of Newly Diagnosed Multiple Myeloma Patients

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Objective. To investigate the efficacy and safety of the treatment of the newly diagnosed multiple myeloma (MM) patients with the therapy of subcutaneous (subQ) administration of bortezomib and dexamethasone plus thalidomide (VTD) regimen. *Methods*. A total of 60 newly diagnosed MM patients were analyzed. 30 patients received improved VTD regimen (improved VTD group) with the subQ injection of bortezomib and the other 30 patients received conventional VTD regimen (VTD group). The efficacy and safety of two groups were analyzed retrospectively. *Results*. The overall remission (OR) after eight cycles of treatment was 73.3% in the VTD group and 76.7% in the improved VTD group ($P > 0.05$). No significant differences in time to 1-year estimate of overall survival (72% versus 75%, $P = 0.848$) and progression-free survival (median 22 months versus 25 months; $P = 0.725$) between two groups. The main toxicities related to therapy were leukopenia, neutropenia, thrombocytopenia, asthenia, fatigue, and renal and urinary disorders. Grade 3 and higher adverse events were significantly less common in the improved VTD group (50%) than VTD group (80%, $P = 0.015$). *Conclusions*. The improved VTD regimen by changing bortezomib from intravenous administration to subcutaneous injection has noninferior efficacy to standard VTD regimen, with an improved safety profile and reduced adverse events.

1. Introduction

Bortezomib, the first potent therapeutic proteasome inhibitor, has been suggested as a standard care in patients with newly diagnosed and relapsed multiple myeloma (MM) [1]. Bortezomib is associated with high efficacy response rate when it is used as induction therapy before high-dose therapy (HDT) plus autologous stem cell transplantation (ASCT) [2, 3]. Intravenous injection is the standard route of bortezomib administration; the recommended dose and schedule of bortezomib is 1.3 mg/m^2 on days 1, 4, 8, and 11 of a 21-day cycle, for up to eight cycles, administered by 3–5/second intravenous (IV) bolus; this dose and schedule is active and well tolerated [4, 5]. However, IV administration requires repeated intravenous access or insertion of long-term central venous access devices and is usually associated with some serious adverse events [6].

Recently, two clinical trials have confirmed that subcutaneous (subQ) administration of bortezomib represents a good option to optimize the use of bortezomib for MM patients and results in a more convenient route that is at least as effective as the IV route [7, 8]. A phase I study conducted by French Francophone Myeloma Intergroup compared the pharmacokinetics and pharmacodynamics, safety and efficacy of IV, and subQ administration of bortezomib in patients with relapsed and/or refractory MM. The results demonstrated that subQ administration of bortezomib was possible because there were no differences in overall systemic availability and pharmacodynamic activity, toxicity profiles, and response rates in MM [7]. An international, multicenter,

and randomized phase III study was then performed to confirm the safety and efficacy of this new route; 222 patients were randomly assigned to receive up to eight 21-day cycles of subcutaneous or intravenous bortezomib; the results confirmed that subQ bortezomib was not inferior to standard IV route, with even an improved safety profile and lower incidence of severe adverse events [8].

Bortezomib was approved for the treatment of MM in 2003, and since then several bortezomib-based combination therapies have developed. Regimens that have combined bortezomib with corticosteroids, alkylating agents, and immunomodulation drugs have resulted in high response rates [9]. The triplet combination of bortezomib and thalidomide plus dexamethasone (VTD) has proved to be a highly effective and well tolerated induction therapy for MM patients who were eligible for HDT-ASCT [10–12]. However, no relevant literatures were found regarding the subQ bortezomib-based VTD regimen as induction therapy for patients with MM. Therefore, the current single-center, retrospective study was designed to investigate the efficacy and safety of improved VTD regimen with the subQ administration of bortezomib in the treatment of the newly diagnosed MM patients.

2. Materials and Methods

2.1. Patients. A total of 60 patients with newly diagnosed MM from January 2009 to June 2013 who did not take part in a clinical trial were included in this study. According to practice guidelines at our center, patients were not excluded from VTD therapy on the basis of creatinine clearance rate or dialysis dependence. Patients with grade 2 or worse peripheral neuropathy were offered alternative therapy; 30 of them received improved VTD regimen (improved VTD group) with the subQ injection of bortezomib, and the other 30 patients received conventional VTD regimen (VTD group). The study was approved by the institutional review board of our hospital. Informed consents were obtained from the patients prior to this study.

2.2. Study Design. All the patients belonged to International Stage System (ISS) I–III in which transplantation patients were excluded or the patients refused receiving transplantation therapy. In both groups, all the patients were treated with VTD regimen as induction therapy. Bortezomib, at a dose of 1.3 mg/m^2, on days 1, 4, 8, and 11, was administered by subcutaneous (improved VTD group) or intravenous injection (VTD group). Subcutaneous injections were administered at 2.5 mg/mL (3.5 mg bortezomib reconstituted with 1.4 mL normal saline) to limit the volume injected. Subcutaneous injection sites were the thighs or abdomen, which were rotated for successive injections. Intravenous injections were administered at a concentration of 1 mg/mL (3.5 mg bortezomib reconstituted with 3.5 mL normal saline) as a 3–5 s intravenous push. Oral thalidomide was given every day at a dose of 200 mg/d. Oral dexamethasone was given at a dose of 40 mg/d on days 1 through 4 and days 9 through 12. Each cycle was repeated every 21 d for up to 8 cycles. Treatment was suspended when drug-related grade 4 hematological toxic effects or grade 3-4 nonhematological toxic effects occurred.

2.3. Assessments. All patients were assessed for response and progression according to the international uniform response criteria for multiple myeloma (IMWG) [13] every 3 weeks. Baseline evaluations including physical examination, blood counts, hepatic and renal function tests, bone marrow aspirate and biopsy, serum and urine protein electrophoreses, and quantitation of serum immunoglobulin and urinary light chains and β_2-MG were performed before each cycle. Interphase FISH were performed to identify cytogenetic abnormalities. Toxicities were evaluated according to National Cancer Institute Common Terminology Criteria for Adverse Events Version 3.0. Patients receiving at least two cycles of VTD regimen were included in the toxicity evaluation.

2.4. Statistical Analysis. SPSS version 17.0 software (Chicago, IL) was used for data analysis. The efficacy was evaluated by chi square test. Survival analysis was performed with life table and Kaplan-Meier survival curve. P value less than 0.05 was considered statistically significant.

3. Results

3.1. Patient Characteristics. A total of 60 patients with MM were recruited in this retrospective analysis. 30 patients received VTD therapy and the other 30 patients received improved VTD therapy. Their demographic and baseline characteristics are summarized in Table 1. Among these patients, 35 were males and 25 were females; the median age was 56 years (range, 31 to 72 years). IgG MM was found in 26 patients, IgA in 16 patients, IgD in 5 patients, and light chain MM in 13 patients. 12 patients were stage 1, 33 were stage II, and 15 were stage III. The baseline characteristics were similar in the two groups.

3.2. Efficacy. In both groups, patients received a median of six treatment cycles (range, four to eight). Overall remission (OR) after eight-cycle treatment was 73.3% in the VTD group (22 of 30 patients) and 76.7% in the improved VTD group (23 of 30 patients), including 4 patients (13.3%) getting complete remission (CR), 10 (33.3%) very good partial response (VGPR), and 8 (26.7%) partial remission (PR) in the VTD group and 3 patients (10%) getting CR, 11 (36.7%) VGPR, and 9 (30%) PR in the improved VTD group (Table 2). There was no statistical difference between the two groups ($P > 0.05$).

3.3. Prognosis. After a median follow-up of 24 (range, 3–36) months, we noted no significant difference in 1-year estimate of overall survival (72% versus 75%, $P = 0.848$) and progression-free survival (median 22 months, 95% CI 7.16–36.8, versus 25 months, 95% CI 9.08–36.1; $P = 0.725$) between VTD group and improved VTD group (Figure 1).

3.4. Safety. All patients experienced at least one adverse event. The main toxicities related to therapy in the two groups

Table 1: Patient demographics and baseline characteristics ($n = 60$).

Characteristic	VTD group ($n = 30$)	Improved VTD group ($n = 30$)	P value
Sex (male/female)	18/12	17/13	0.793
Median age (years, range)	54 (31–67)	57 (34–72)	0.712
Myeloma type			
IgG	12 (40%)	14 (46.7%)	0.602
IgA	9 (30%)	7 (23.3%)	0.559
IgM	2 (6.7%)	3 (10%)	0.640
Light chain	7 (23.3%)	6 (20%)	0.754
ISS stage			
I	6 (20%)	6 (20%)	1.000
II	18 (60%)	15 (50%)	0.436
III	6 (20%)	9 (30%)	0.371
Cytogenetics			
Diploid	15 (50%)	13 (43.3%)	0.605
Hyperdiploid	6 (20%)	7 (23.3%)	0.754
Nonhyperdiploid	6 (20%)	8 (26.7%)	0.542
Hypodiploid	3 (10%)	2 (6.7%)	0.640
Interphase FISH			
t(4;14)	15 (50%)	12 (40%)	0.436
del(17p13)	9 (30%)	14 (46.7%)	0.184
t(11;14)	6 (20%)	4 (13.3%)	0.488
Hemoglobin (g/L)	103 (71–144)	109 (73–159)	0.677
Albumin (g/L)	37.5 (22–47)	36 (24–43)	0.820
β_2 microglobulin (mg/L)	3.9 (2.2–16.9)	4.3 (2.3–18.3)	0.754
Platelets ($\times 10^9$/L)	243.4 (98.2–602.1)	251.7 (79.3–533.2)	0.501
Creatinine (mg/dL)	1.6 (0.4–3.7)	1.7 (0.2–4.1)	0.835

Table 2: Response to VTD regimen in each group.

Response (n, %)	After 8 cycles		P value
	VTD group ($n = 30$)	Improved VTD group ($n = 30$)	
OR	22 (73.3%)	23 (76.7%)	0.766
CR	4 (13.3%)	3 (10%)	0.688
VGPR	10 (33.3%)	11 (36.7%)	0.787
PR	8 (26.7%)	9 (30%)	0.774
MR	4 (13.3%)	4 (13.3%)	1.000
SD	3 (10%)	3 (10%)	1.000
PD	1 (3.3%)	0	0.313
Not evaluable	0	0	1.000

OR (CR + VGPR + PR): overall response; CR: complete response; VGPR: very good partial response; PR: partial response; MR: minimal response; SD: stable disease; PD: progressive disease; VTD: bortezomib and thalidomide plus dexamethasone.

included leukopenia, neutropenia, thrombocytopenia, asthenia, fatigue, and peripheral sensory neuropathy (Table 3). Most adverse events were grades 1-2. Grade 3 and higher adverse events were reported in 24 of 30 (80%) patients in the VTD group and 15 of 30 (50%) in the improved VTD group ($\chi^2 = 5.943$, $P = 0.015$), with 8 (26.7%) and 3 (10%) discontinuing and 8 (26.7%) and 2 (6.7%) needing bortezomib dose reductions because of adverse events, respectively. Three of 30 (10%) patients in improved VTD group had one or more subcutaneous injection-site reaction reported, which resulted in a bortezomib dose modification in two (6.7%) patients (discontinuation or dose withholding). The most common reaction was injection-site erythema. No death related to therapy was reported in this study.

4. Discussion

In recent years, the outcome of MM patients has been significantly improved due to the discovery of novel antimyeloma agents together with a better knowledge of the pathophysiology of the disease. Among them, the proteasome inhibitor bortezomib (Velcade) represents an excellent drug that has quickly moved from the bench to the bedside and exhibits a powerful antimyeloma activity. Nowadays, bortezomib-based therapies are suggested as standards of care in patients with newly diagnosed and relapsed multiple myeloma [1]. In addition, abundant studies about the efficacy of bortezomib

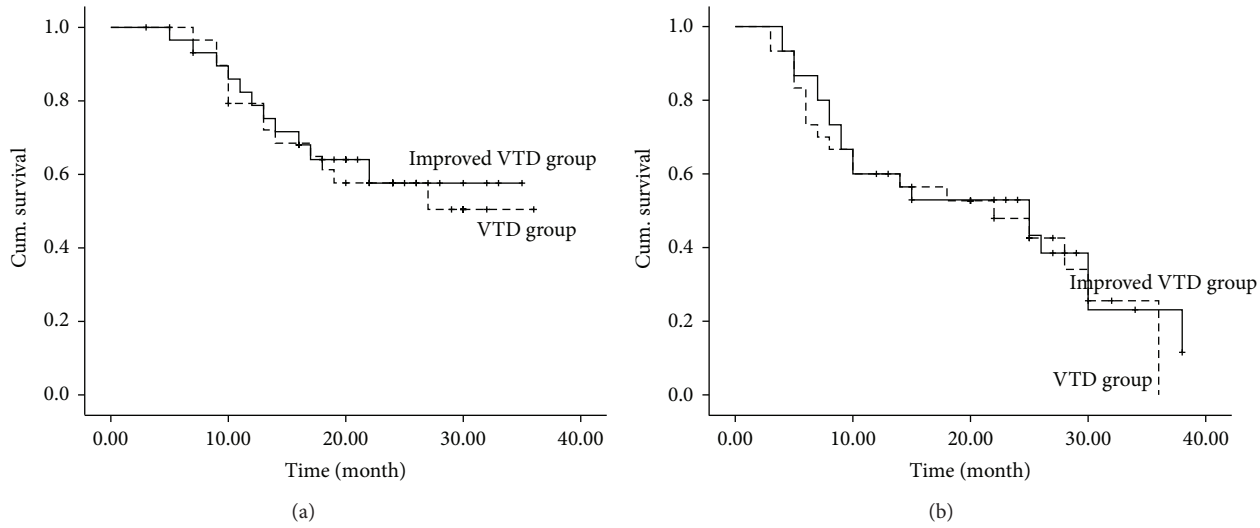

Figure 1: Kaplan-Meier curve of overall survival (a) and progression-free survival (b) after bortezomib and thalidomide plus dexamethasone (VTD) induction therapy.

Table 3: Incidence of adverse events related to VTD regimen in each group.

Events (n, %)	VTD group ($n = 30$)		Improved VTD group ($n = 30$)		χ^2	P^*
	All grades	Grade ≥ 3	All grades	Grade ≥ 3		
Leukopenia	27 (90%)	18 (60%)	22 (73.3%)	9 (30%)	5.455	**0.020**
Neutropenia	25 (83.3%)	13 (43.3%)	22 (73.3%)	12 (40%)	0.069	0.793
Thrombocytopenia	27 (90%)	19 (63.3%)	24 (80%)	16 (53.3%)	0.617	0.432
Anemia	15 (50%)	5 (16.7%)	12 (40%)	5 (16.7%)	0.000	1.000
Peripheral sensory neuropathy	17 (56.7%)	6 (20%)	10 (33.3%)	3 (10%)	1.176	0.278
Diarrhoea	11 (36.7%)	3 (10%)	10 (33.3%)	1 (3.3%)	1.071	0.301
Neuralgia	3 (10%)	1 (3.3%)	2 (6.7%)	0	1.107	0.313
Pyrexia	2 (6.7%)	0	1 (3.3%)	0	0.000	1.000
Nausea	18 (60%)	5 (16.7%)	10 (33.3%)	3 (10%)	0.577	0.448
Vomiting	14 (%)	4 (13.3%)	10 (33.3%)	3 (10%)	0.162	0.688
Asthenia	24 (80%)	5 (16.7%)	17 (56.7%)	3 (10%)	0.577	0.448
Constipation	12 (40%)	2 (6.7%)	9 (30%)	0	2.069	0.150
Fatigue	26 (86.7%)	11 (36.7%)	21 (70%)	4 (13.3%)	4.356	**0.037**
Weight decreased	3 (10%)	0	1 (3.3%)	0	0.000	1.000
Pneumonia	4 (13.3%)	0	2 (6.7%)	0	0.000	1.000
Eye disorders	2 (6.7%)	0	0	0	0.000	1.000
Renal and urinary disorders	21 (70%)	4 (13.3%)	13 (13.3%)	1 (3.3%)	1.964	0.161
Skin and subcutaneous tissue disorders	4 (13.3%)	1 (3.3%)	2 (6.7%)	0	1.107	0.313
Hepatobiliary disorders	6 (20%)	3 (10%)	4 (13.3%)	2 (6.7%)	0.218	0.640
Psychiatric disorders	1 (3.3%)	0	0	0	0.000	1.000

* shows that grade 3 and higher adverse events were compared by chi square test. VTD: bortezomib and thalidomide plus dexamethasone; improved VTD: subQ bortezomib and thalidomide plus dexamethasone.

as a single agent or in combination with other agents in relapsed and/or refractory as well as in newly diagnosed myeloma patients have emerged, and all data have contributed to confirming bortezomib as one of the key drugs of the backbone treatment of myeloma patients [9].

The triplet combination of bortezomib and thalidomide plus dexamethasone (VTD) regimen was one of the highly effective and well tolerated induction therapies for MM patients. In our study, the overall response rate was 73.3% with VTD regimen therapy, including 13.3% CR, 33.3% VGPR, and 26.7% PR in newly diagnosed MM patients. Previous phase 3 study by the Italian Group for Adult Hematologic Diseases (GIMEMA) compared VTD with TD as induction therapy in newly diagnosed patients [14]. The results showed that VTD produced significantly higher response rates than TD both after induction (94% overall

rate, including a 62% VGPR rate and a 32% CR/near-CR rate, versus 79% overall rate, including a 29% VGPR rate and a 12% CR/near-CR rate) and after transplantation (a 76% VGPR rate, including a 55% CR/near-CR rate, versus a 58% VGPR rate, including a 32% CR/near-CR rate). Combination of bortezomib with other immunomodulatory drugs and dexamethasone as induction therapy in newly diagnosed patients with MM also has been demonstrated in 2 studies of the combination of bortezomib, the thalidomide analog lenalidomide, and dexamethasone, which produced a 100% overall response rate, including a 75% VGPR rate and a 40% CR/near-CR rate [15, 16]. These results and ours demonstrate that VTD regimen is highly active and well tolerated as induction therapy in patients with MM.

The primary goal of the retrospective study was to compare the subQ bortezomib-based VTD regimen and conventional VTD regimen as induction therapy for patients with MM. Subcutaneous administration of bortezomib has been shown to be noninferior to the standard intravenous route of delivery in patients with relapsed multiple myeloma and has an improved systemic safety profile [7, 8]. Recently, subQ bortezomib-based regimen has emerged and is considered as a promising alternative to intravenous administration, particularly in patients with poor venous access or at increased risk of side-effects [17–19]. In this study, the improved VTD regimen by changing bortezomib from intravenous administration to subcutaneous injection showed noninferior efficacy to standard VTD regimen. We recorded similarity between groups across all efficacy endpoints, including rates of OR and CR and very good PR after eight cycles. This finding accords with previous results showing similar response rates of MM patients treated with improved bortezomib, adriamycin, and dexamethasone (PAD, 61.1%) with the subQ injection of bortezomib and conventional PAD regimen (57.1%) [17]. We also found that there were no significant differences in time to 1-year estimate of overall survival (72% versus 75%, $P = 0.848$) and progression-free survival (median 22 months, 95% CI 7.16–36.8, versus 25 months, 95% CI 9.08–36.1; $P = 0.725$) between VTD group and improved VTD group. Taken together, this study provided further information that subQ administration of bortezomib is feasible and could contribute to optimizing the management of bortezomib in the treatment of myeloma patients.

We also provided important findings about the toxic effects of bortezomib in the subcutaneous group and intravenous group. The main toxicities related to therapy in the two groups were leukopenia, neutropenia, thrombocytopenia, asthenia, fatigue, and peripheral sensory neuropathy. Subcutaneous administration had an improved systemic safety profile compared with intravenous delivery, with lower rates of grade 3 or higher adverse events, and with fewer bortezomib dose reductions and discontinuations because of adverse events. Subcutaneous administration also had acceptable local tolerability; only 3 patients developed one or more subcutaneous infection-site reactions reported as an adverse event, as resulted in a bortezomib dose modification in two patients. All of these results confirmed that subQ bortezomib-based VTD regimen was not inferior to IV route, with even an improved safety profile.

In conclusion, VTD is highly active and well tolerated induction therapy for patients with MM. The improved VTD regimen by changing bortezomib from intravenous administration to subcutaneous injection has noninferior efficacy to standard VTD regimen and may become the front-line therapy for the newly diagnosed MM patients. Further studies in larger populations and a long follow-up are warranted to confirm the result.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Rituximab as Single Agent in Primary MALT Lymphoma of the Ocular Adnexa

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Ocular Adnexal Lymphomas are the first cause of primary ocular malignancies, and among them the most common are MALT Ocular Adnexal Lymphomas. Recently systemic immunotherapy with anti-CD20 monoclonal antibody has been investigated as first-line treatment; however, the optimal management for MALT Ocular Adnexal Lymphomas is still unknown. The present study evaluated retrospectively the outcome of seven consecutive patients with primary MALT Ocular Adnexal Lymphomas, of whom six were treated with single agent Rituximab. All patients received 6 cycles of Rituximab 375 mg/mq every 3 weeks intravenously. The overall response rate was 100%; four patients (67%) achieved a Complete Remission, and two (33%) achieved a partial response. In four patients an additional Rituximab maintenance every 2-3 months was given for two years. After a median follow-up of 29 months (range 8–34), no recurrences were observed, without of therapy- or disease-related severe adverse events. None of the patients needed additional radiotherapy or other treatments. Rituximab as a single agent is highly effective and tolerable in first-line treatment of primary MALT Ocular adnexal Lymphomas. Furthermore, durable responses are achievable with the same-agent maintenance. Rituximab can be considered the agent of choice in the management of an indolent disease in whom the "quality of life" matter is of primary importance.

1. Introduction

Ocular Adnexal Lymphomas (OALs) are a heterogeneous group of lymphoproliferative neoplasms involving the orbital anatomic region and its structures: lacrimal glands, extraocular muscles, conjunctiva, eyelids, and the orbit itself. They are the main cause of primary ocular malignancies, accounting for more than 50% of cases [1], and represent about 1-2% of Non Hodgkin Lymphomas (NHL) and 8% of Extranodal NHLs. Extranodal Marginal Zone Lymphoma (MALT lymphoma) is the most common histology of primary OALs (50–80% of cases), followed by Follicular Lymphoma (10–20%), Diffuse Large B-cell Lymphoma (8%), and other less common low grade B-cell NHL, with rare incidence of aggressive, T-cell, and Hodgkin lymphomas. The great majority (92%) of Extranodal Marginal Zone OALs are primarily ocular, while other histologies, in particular high grade diseases, in many cases involve ocular structures primarily or secondarily [2]. Recent data about OALs show that incidence has been increasing over the last decades [3, 4]. The postulated origin of these neoplasms is the postgerminal-center memory B cell, which has the capacity to differentiate into marginal zone cells and plasma cells.

Treatment, for lymphoproliferative disorders involving ocular adnexa, may be widely different. In fact, while high grade or multicentric forms of lymphomas invariably need

Table 1: Demographic data, tumor features, and response, in 6 patients with ocular adnexal lymphoma treated with Rituximab immunotherapy.

No.	Age, sex	Eye	Stage	Location	RTX cycles	Interim response	RTX response	RTX maintenance	Survival status	FU months	Final status
1	54/F	OS	IE	C	6	SD	CR	No	Alive	34+	CR
2	62/M	OS	IE	C	6	n.e.	CR	Yes	Alive	27+	CR
3	59/F	OD	IE	C	6	n.e.	CR	Yes	Alive	31+	CR
4	67/F	OS/OD	IE	C	6	n.e.	PR	No	Dead*	8	—
5	54/M	OS	IE	O	6	CR	CR	Yes	Alive	31+	CR
6	37/F	OD	IE	O	6	PR	PR	Yes	Alive	9+	PR

M: male; F: female; OS: left eye; OD: right eye; C: conjunctive; O: orbit; SD: stable disease; PR: partial response; CR: complete response; n.e: not evaluated; RTX: rituximab; FU: follow-up.
*Lung carcinoma.

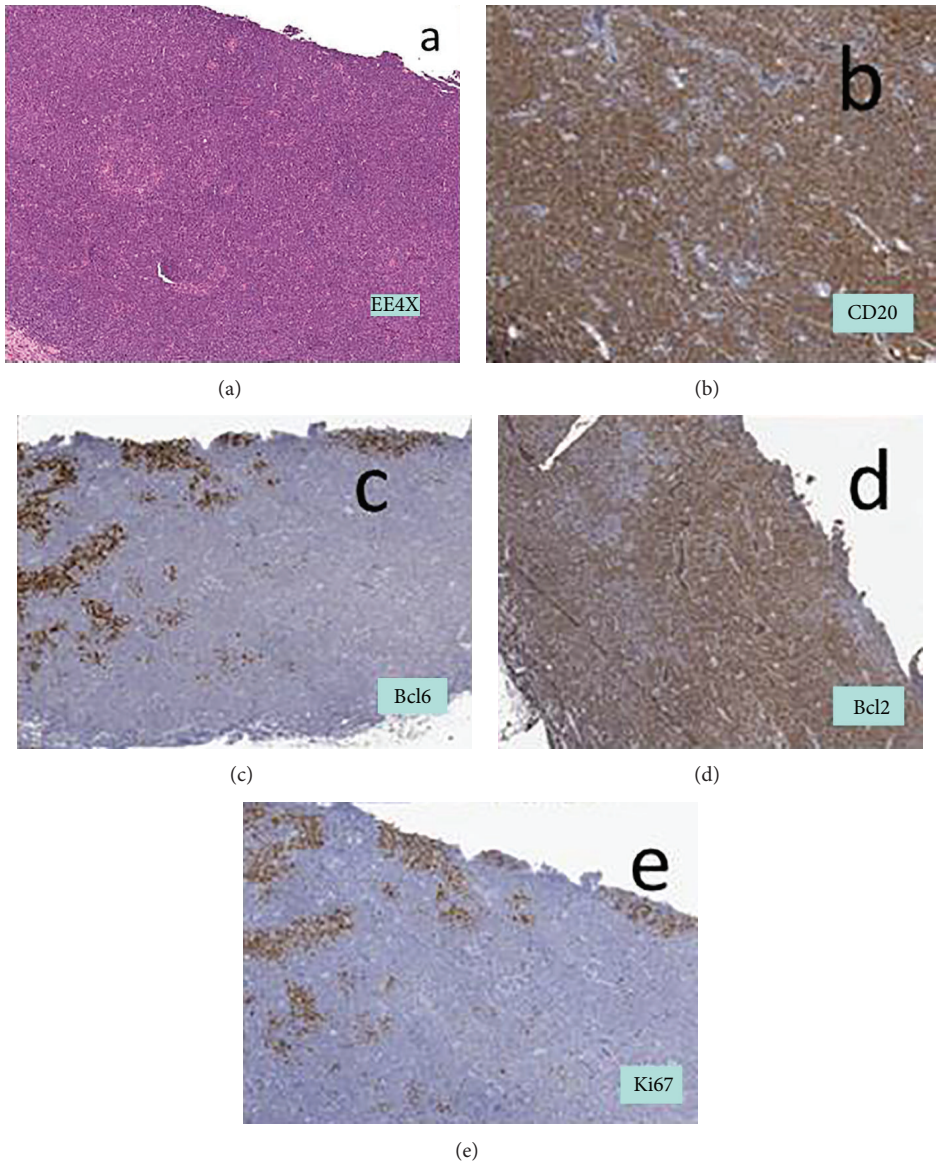
systemic polychemotherapy, indolent and localized lymphomas like MALT OALs, which represent the vast majority of the cases, may not need an intensive systemic treatment. In the past decades many treatments for MALT OALs were used: surgical resection, antibiotic therapy, cryotherapy, radiotherapy, and interferon alpha. More recently immunotherapy with Rituximab emerged as an interesting option, because of its safe toxicity profile and good tolerability together with the chance of durable remissions. However, the real value of Rituximab immunotherapy in primary MALT OALs is not well established yet. For this reason, we evaluated the efficacy of systemic Rituximab immunotherapy in 7 consecutive patients with primary MALT OAL.

2. Patients and Methods

From 2004 to 2014 we observed 11 consecutive OALs. Of these, 7/11 (63% of cases) were MALT lymphomas, 2/11 (18%) were Mantle Cell Lymphomas, 1/11 (9%) was a Follicular Lymphoma, 1/11 (9%) was a Marginal Zone B-cell lymphoma. We included in this analysis 7 consecutive patients with primary histologically diagnosed CD20+ MALT OALs according to the WHO 2008 classification [5], Ann Arbor staging system IE, treated with Rituximab immunotherapy alone between March 2012 and December 2014. One of these patients, showing an increased uptake in PET scans, was excluded from the study because of a relatively aggressive bilateral disease and underwent treatment with R-COMP polychemotherapy. None of the patients enrolled was previously treated. For each of the 6 eligible patients we recorded age, sex, laterality, affected tissue, presenting signs and symptoms, serologic markers, dose and response to Rituximab treatment, follow-up period, complications, and survival status. At the diagnosis in all patients an incisional or excisional biopsy with immunohistochemical staining for histopathologic definition was performed. In Figure 1, we showed characteristic diffuse infiltrate of lymphoid element surrounding reactive follicles. Moreover, a complete ophthalmic examination, a Total Body Computer Tomography (CT) scan, a Positron Emission Tomography (PET) scan, and an Esophagogastroduodenoscopy and Colonscopy were performed to exclude any systemic involvement. To define the tumor extension and its relationship with close structures, a Magnetic Resonance Imaging (MRI) of the orbital region was also performed. Bone marrow biopsy was not performed since previous studies have demonstrated any benefit in the staging of MALT OALs [6]. All patients received six cycles of systemic Rituximab immunotherapy at a dose of 375 mg/mq intravenously, every 3 weeks. Three patients (50%) were evaluated with an interim MRI scan after three cycles. In all patients after the sixth cycle the response to treatment was assessed with an MRI and a PET scan to define three grades of response: complete, partial, or stable disease. Response to treatment was evaluated on the basis of clinical, radiologic, and pathologic criteria, with the definition of a complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) referring to the international response criteria for malignant lymphoma [7]. Response was evaluated at the end of treatment program (after 6 courses).

3. Results

In Table 1 patients and disease features, treatment, and outcome are summarized. Median age was 57 years (range 37–67 years), four females, and two males were enrolled, with a female/male ratio of 4/2. Five patients (83%) presented with unilateral disease, and one (17%) with bilateral involvement. In four patients (66%) the disease involved the conjunctiva, and in two patients (33%) it had an orbital localization. In none of the cases there was presence of systemic disease. The most common presenting signs and symptoms were ocular swelling (four patients, 67%), conjunctival erythema (17%), and xerophthalmia (17%). All the patients were diagnosed with biopsy-proven histological examination which resulted in CD20+. The diagnosis in all patients was Mucosa-Associated Lymphoid Tissue OAL. None but one of the patients was previously treated for their ocular disease. The pretreated patient had received interferon α-2b, that was rapidly discontinued (after few days) for intolerance. All patients received 6 cycles of Rituximab systemic immunotherapy at the standard dose of 375 mg/mq every 21 days. With the exception of one patient having a Varicella Zoster Virus (VZV) reactivation (Ramsay Hunt syndrome) treated with acyclovir per os, no systemic or ocular relevant side effects were observed. Of the three patients who underwent an interim evaluation by MRI scan.



(a) (b) (c) (d) (e)

Figure 1: Characteristic diffuse infiltrate of lymphoid element with small nuclei (a) positive to immunohistochemical staining for CD20 (b) and bcl2 (d) and negative for bcl6 (c) with low ki67 (e). The infiltrate surrounds reactive follicles evidenced by positivity for bcl6 and negativity for bcl2 associated with high ki67.

As showed in Figure 2, one had a complete response (CR), the second had a partial response (PR), and in the third case a stable disease (SD) was demonstrated. After the sixth Rituximab cycle, four patients (67%) achieved a CR, and the remaining two patients (33%) achieved a PR. On the whole, all the six patients were responders to Rituximab treatment. After the completion of this treatment, four patients started a maintenance therapy with Rituximab 375 mg/mq every 2-3 months for two years. Of them, three are still in CR and one maintains a PR. Rituximab maintenance was well tolerated in all patients, except one case who presented herpetic keratitis (he was the same patient who had had the VZV reactivation). None of the patients underwent local radiotherapy. After a median follow-up of 29 months (range 8–34), no recurrences of MALT lymphoma were observed, nor treatment or disease-related deaths. Five of the six patients are alive at the time of this analysis (January 2015); one patient died because of lung cancer relapse and could not start Rituximab maintenance; this patient had achieved a PR of its OAL. Maintenance treatment with Rituximab is still ongoing in 4/5 alive patients.

4. Discussion

Primary localized MALT OALs are malignancies having indolent behavior, usually associated with a favorable prognosis, rare lymphoma-related deaths, and a non- or oligosymptomatic course. Thus, the treatment strategy should be chosen considering both efficacy profile and

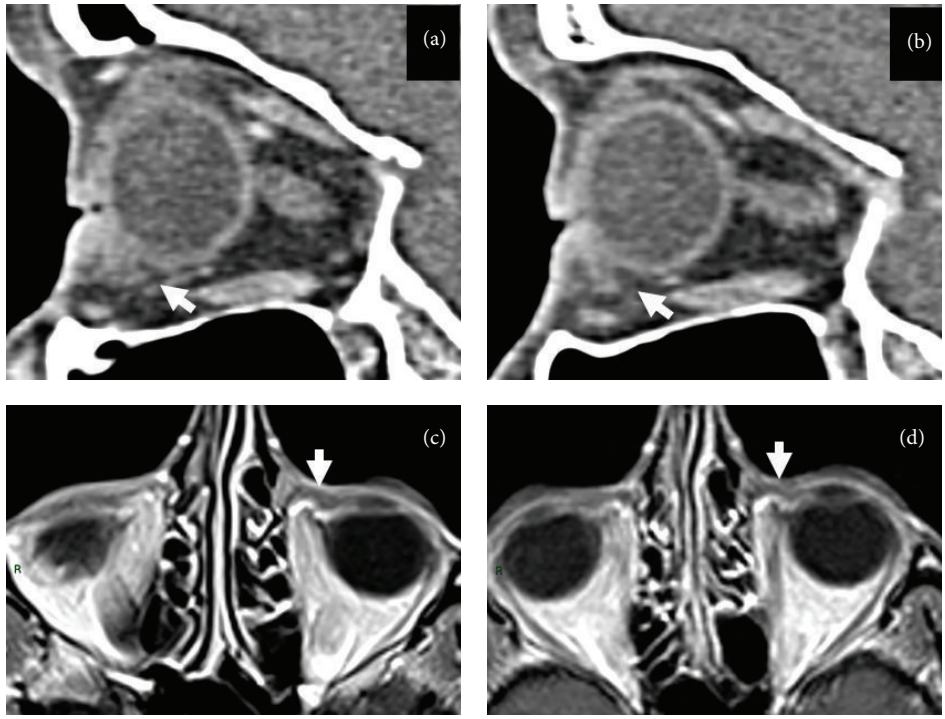


Figure 2: Imaging signs of B cell lymphoma response to therapy. ((a) and (b)) Computed tomography sagittal reformats show focal thickening of the anteroinferior peribulbar conjunctiva on the left side (white arrow in (a)). Compared to the same site in the right orbit (arrow in (b)) where peribulbar hypodense fat tissue is present. ((c) and (d)) Magnetic resonance axial postgadolinium T1-weighted images before (c) and after (d) treatment. Note the size reduction of the focal peribulbar tissue on the left medial conjunctiva (white arrows in (c) and (d)).

toxic effects. Beside conventional treatments like surgery, chemotherapy, and radiotherapy, associated with potential systemic and local damage, other less toxic strategies have been studied, including intralesional injection of Interferon α-2b, brachytherapy, and antibiotic therapy. In particular, surgical excision alone as treatment of OALs is followed by local relapse and by disseminated extraocular disease [8–10]; therefore, the role of surgery is currently limited only to diagnostic biopsy.

Radiotherapy plays an intriguing role in the treatment of OALs. It has been proven that radiotherapy is capable of inducing a local control rate of the disease up to 100% of cases regardless of the histologic subtype of the lymphoma and a low recurrence rate ranging between 0% and 15%. Therefore, it is considered to be the standard treatment for low grade OALs localized to the orbit [11–15]. Moreover, in 17%–65% of patients, a lead shield to protect the cornea was used. Depending on the studies median dose of radiotherapy ranged between 24 and 30.6 Gy and the amount of Gy per fraction varied between 1.5 and 2.5 Gy depending on the study [6, 16–23].

Generally single agent chemotherapy such as chlorambucil or purine analogs (fludarabine, cladribine, and pentostatin) or low toxicity combined regimens such as CVP (cyclophosphamide, vincristine, and prednisone) are utilized for the chemotherapeutic treatment of OALs patients who have or not systemic involvement. The adjunct of chemotherapy to radiotherapy did not add any benefit, and the toxicities rates were similar between the two treatment regimens [9, 24]. Considering the proposed role of Chlamidia psyttaci in the pathogenesis of OAL, an original approach in the treatment of these types of Lymphoma has been that of using antibiotic treatment directed against *Chlamydia psittaci*. This type of antibiotic treatment was firstly proposed by Ferreri et al. who showed an objective clinical response in 80% of treated patients with doxycycline [25]. This result was confirmed by Abramson et al. [26]. On the contrary, Grünberger and colleagues [27] did not observe any positive results in their patients. Finally a further study reported that oral doxycycline led to a positive clinical response in 64% of *Chlamydia psittaci* DNA-positive and 38% DNA-negative OALs [28] leading to the conclusion that results obtained in OALs with doxycycline are variable.

In a recent review on the use of antibiotic therapy in nongastrointestinal MALT lymphoma [29] the cumulative results obtained with the use of doxycycline 100 mg BID for 21 days in a total of 8 studies [25–28, 30–33] were reported. Only 3 of these studies were prospective [25, 28, 30], and one was a case report [33]. Overall, in the prospective studies 70 newly diagnosed OALs were accrued, while the retrospective studies have accrued 58 patients. A further study reported in this review was a prospective study utilizing in 11 OALs patients Clarithromycin 500 mg BID for 6 months [34]. Overall, in these 9 studies 23 patients (18%) achieved Complete Remission, 36 (27%) had partial remission, 55 (42%) had stable disease, and 8 (6%) had a progressive disease accounting for an overall response rate of 45%. Very recently, a complete response was obtained by the use of clarithomycin

500 mg twice per day for 4 weeks in a OALs who refused conventional oncologic therapy and tested negative for all potential bacterial causes of MALT lymphoma proposed so far [35].

In the last years the efficacy of systemic single agent Rituximab immunotherapy has been emphasized in the management of primary MALT OALs, as second-line [36] or first-line [37] treatment. However, because of the rarity of the disease, the available data are not uniform. Larger case series attempt to define treatment outcomes with different agents [23, 38]. Meanwhile the studies available about the use of upfront Rituximab as a single agent are very few [39, 40]. Furthermore, no data were available about the possible use of Rituximab maintenance during the follow-up of MALT OALs. Recently Ardeshna et al. [41] have demonstrated an improved Progression Free Survival (PFS) in indolent lymphomas receiving a 2-year maintenance treatment with Rituximab versus no treatment.

Taking into account the small number of patients enrolled, the first aim of our report is to strengthen the excellent response rate of untreated primary MALT OALs to single agent Rituximab demonstrated in literature. Overall response rate (ORR) was, in fact, 100%, and the quality of response was high for the majority of the cases, reaching a CR in four patients (67%) and a PR in two patients (33%), without recurrence. In our study, differently from other reports, all patients were treated with the same induction schedule (6 cycles of Rituximab 375 mg/mq every 3 weeks).

The second aim is to explore the usefulness of Rituximab maintenance in this specific clinical setting, not investigated yet in any report. In our case series four of the six patients were, after Rituximab induction, subsequently maintained with Rituximab every 2 or 3 months. After a median follow-up period of 29 months from the start of therapy and of 21 months from the start of maintenance treatment, we observed no serious adverse events and all patients maintained the achieved response. A limit of our study is the relatively short follow-up together with the limited enrollment; however, it is the first description of clinical outcome in localized primary MALT OALs patients treated with first-line single agent Rituximab followed by same-agent maintenance.

At the present, the main question regarding the optimal management for localized primary MALT OALs cannot be answered yet, since no prospective randomized trials comparing different upfront treatments have been conducted.

As a local approach, surgical excision can be a weapon to treat encapsulated tumors. However, the risk of an incomplete resection is too high and generally not acceptable according to most authors [42, 43].

Involved-field radiotherapy (IFRT) is the current standard of care and has been widely studied in MALT OALs. In the literature good response patterns (OR 85–100%) and durable local control are reported, though accompanied by ocular short- and long-term adverse effects (conjunctivitis, cataract, xerophthalmia, retinopathy, corneal damage, and vision loss) [16, 21, 44]. As reported also in the study by Sasai et al. [9], IFRT seems associated with a considerable risk of systemic recurrence, while a minor risk is seen with Rituximab treatment. The risk of systemic relapse is higher in bilateral ocular presentation of MALT OALs [45], and this seems to suggest a questionable usefulness of IFRT in bilateral disease. Moreover, there is no universally accepted radiation schedule for patients with OAL, and controversy still exists regarding the optimal radiation dose and fractionation (for most authors, comprised between 20 Gy and 30 Gy). Furthermore, retreatment of the same tissue should be avoided, and the "quality of life" matter, in such an indolent disease, should be considered when efficacy is guaranteed by the less toxic treatments.

Also a watchful waiting approach has been studied in patients with asymptomatic localized MALT OALs [46]. Because of the indolent behavior of the disease, this strategy can be considered, according to most authors, only when no other treatments are suitable (e.g., frail elderly patients), and this happens rarely.

Intralesional injection of Interferon α-2b has been attempted, in conjunctival MALT OALs, obtaining good results [47, 48]. Updated follow up results, however, are not available.

Two pilot studies [49, 50] have reported the successful treatment of orbital MALT OALs by intralesional injection of Rituximab; however, long-term effects are not known yet.

In the last years systemic treatments of primary MALT OALs have gained consideration in literature. Several trials studied the efficacy of antibiotic therapy with doxycycline resulting in response rates around 50–60% [51, 52]. However, the wide variability in prevalence of *Chlamydia psittaci* among different geographical regions, and the lower response rate and durability in respect of other treatments make this therapeutic choice not a standard-of-care, especially in western countries.

The use of systemic chemotherapy, with or without immunotherapy, in primary localized MALT OALs, represents a valid alternative in relapsed patients. First-line chemotherapy could be an option but, since there are not prospective trials encouraging it in localized disease, is not commonly recommended because of the high toxicity profile, especially of the anthracycline-containing regimens. A possible effective and well-tolerated agent is oral chlorambucil, alone [53] or in combination with Rituximab [54], but in the literature the duration of response is not better than other local or less toxic agents.

Only few case series are available on the efficacy of single agent Rituximab immunotherapy in primary localized MALT OALs as showed in Table 2 [7, 8, 11, 12, 55–57]. The results of these studies show that systemic immunotherapy could be of primary importance as first-line treatment, because of the high response rates achieved (comparable to those of local radiotherapy), accompanied also by a favorable tolerability profile. Overall, these studies (all including a small population sample) deeply differ in terms of patients population, line of treatment, staging inclusion criteria, and administration schedule. An issue raised from these data indicates a high rate of relapse with Rituximab monotherapy [1, 8]. In our case series we included only nontreated patients, Ann Arbor staging IE, who underwent 6 cycles of intravenous standard-dose Rituximab every 3 weeks, obtaining good response rates similar to those in literature, and without

Table 2: Results by Rituximab in OAL (review of the literature).

	Patients	Diagnosis	Clinical stage	Rituximab dose	Outcome	Longer follows-up (months)
Nückel et al. [36]	2	Relapsed after RT	IE	375 mg/mq once weekly for 4 wks.	1 CR 1 RP	30 and 32
Ferreri et al. [37]	8	5 newly diagnosed 3 relapses	IE (4) IV (4)	375 mg/mq once weekly for 4 wks.	3 CR 2 PR 2 PD 1 SD	Not available
Tuncer et al. [39]	10	Newly diagnosed	IE	375 mg/mq iv every 3 wks. for 6–8 cycles	36% CR 64% PR	31
Zinzani et al. [40]	1	Newly diagnosed	IE	375 mg/mq once weekly for 4 wks.	CR	—
Sullivan et al. [55]	8	Newly diagnosed	—	375 mg/mq once weekly for 4 wks.	5 CR, 2 PR 1 No Res	32
Heinz et al. [56]	1	Newly diagnosed		375 mg/mq once weekly for 4 wks.	CR	—
Mino et al. [57]	10	Newly diagnosed	I-IIE	375 mg/mq every 4 wks. for 6–8 cycles	10 CR	
Present study	6	Newly diagnosed	IE	375 mg/mq every 3 wks. for 6 cycles + maintenance for 2 years	4 CR 2 PR	34

adverse events except from one case of viral reactivation completely resolved with antiviral therapy. We subsequently treated four of the six patients with intravenous maintenance Rituximab every 2-3 months, with sustained response and without serious toxicity. The whole median follow-up period was 29 months. This treatment strategy was never reported before in primary MALT OALs and may overcome the high rate of relapse showed in literature, especially in the control of local disease, which seems to represent a disadvantage in respect of radiotherapy as first-line management.

In conclusion, we consider Rituximab immunotherapy the therapy of choice in the upfront treatment of primary localized MALT OALs. This induction should be followed by Rituximab maintenance. However, perspective trials in the framework of cooperative groups are needed to establish the exact role of Rituximab and the optimal management of these indolent lymphomas. A direct comparison between radiotherapy and immunotherapy should be performed, to answer the question of the best first-line therapy. Since MALT OALs are associated with a favorable prognosis, therapeutic options are equally effective and preserving patients' quality of life should always be preferred.

Conflict of Interests

All authors declare that there is no conflict of interests regarding the publication of this paper.

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