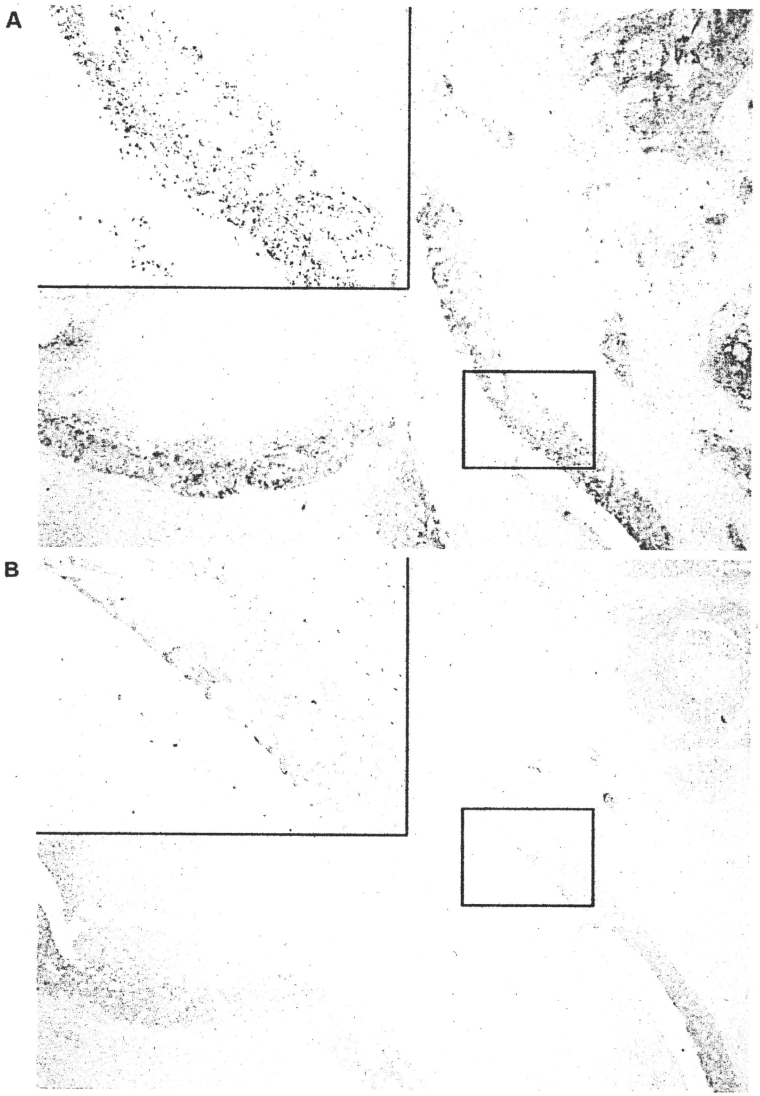

Figure 1. *Representative section of endometrial cancer with immunohistochemical staining of A: WT1 (×40; inset ×200) and B: VEGF (×40; inset ×200). Strong cytoplasmic staining of WT1 and VEGF is observable in the same area of serial sections.*

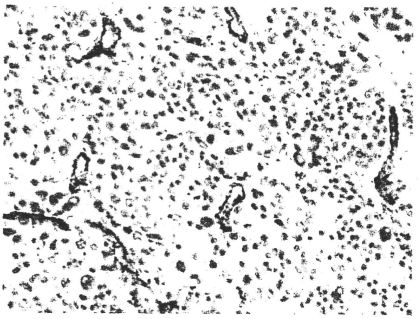

Figure 2. *Representative section of endometrial tumor with immunohistochemical staining of CD31 (×100).*

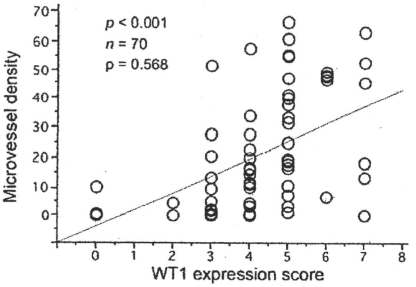

Figure 3. *A positive correlation between microvessel density and WT1 staining score was observed using Spearman's rank correlation coefficient.*

0.32, p=0.001), and that CD105/endoglin-MVD is significantly associated with FIGO stage (p=0.03), tumor cell proliferation estimated by the expression of Ki-67 (p=0.007) in endometrial cancer (20). Ueda *et al.* demonstrated that intratumoral MVD (immunohistochemically using anti-CD34 antibody) was well correlated with histological type (p=0.0415), depth of myometrial invasion (p=0.0176), endometrial invasion (p=0.0354) and pelvic lymph node metastasis (p=0.0354) in uterine cervical cancer (21). Kamat *et al.* showed that high expression of MVD as assessed by CD31 expression was associated with high-stage and grade 3 tumors (p=0.03 and 0.04, respectively) in endometrial cancer (22). In the present study, we found that MVD as assessed by CD31 expression was associated with advanced FIGO stage, myometrial invasion and high-grade histological differentiation. Furthermore, a strong association was found between WT1 staining score and MVD using Spearman's rank correlation coefficient. These results suggest that WT1 may regulate tumor progression and angiogenesis in endometrial cancer.

As a key mediator of angiogenesis, VEGF is tightly regulated at both the transcriptional and post-transcriptional levels. VEGF regulation is complex, since it is up-regulated by hypoxia, growth factors, steroid hormones and transcription factors including WT1 (23). Cash *et al.* showed that WT1 had both transcriptional and post-transcriptional effects on *VEGF* mRNA levels in prostate cell lines (14). Hanson *et al.* found that methyltrienolamine (androgen analog, R1881) increased the transcriptional activation of the *VEGF* promoter by WT1 in LNCaP prostate cancer cells (24). Overall, these reports suggest that WT1 plays an essential role in the transcriptional regulation of *VEGF* in cancer cells. On the other hand, WT1 is essential for kidney development (25-27) and is co-expressed with VEGF in normal kidney cells and in some Wilms' tumors (27-30). Therefore, for both developmental and cancer studies, it is important to elucidate the mechanisms whereby WT1 regulates VEGF and thereby, angiogenesis. In the present study, we also found that WT1 and VEGF were co-expressed in the same area of endometrial cancer tissue. This result suggests that WT1 may regulate expression of VEGF.

These results imply that WT1 plays an important role in endometrial cancer-associated angiogenesis, probably *via* induction of angiogenesis by VEGF. To the best of our knowledge, this may be the first report to demonstrate the positive role of WT1 in endometrial cancer-associated angiogenesis and may prove of great benefit in finding a rational approach to endometrial cancer therapy.

Recently, anti-angiogenic therapy has begun to show promise as an effective treatment strategy for many types of solid tumor. WT1 is also a target for cancer immunotherapy, and this study suggests that a WT1 peptide vaccine therapy might be effective not only as cancer immunotherapy but also as anti-angiogenesis therapy.

In conclusion, tumor-produced WT1, which may regulate the expression of VEGF, is associated with the induction of angiogenesis in endometrial cancer.

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Induction of complete and molecular remissions in acute myeloid leukemia by Wilms' tumor 1 antigen-targeted dendritic cell vaccination

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Active immunization using tumor antigen-loaded dendritic cells holds promise for the adjuvant treatment of cancer to eradicate or control residual disease, but so far, most dendritic cell trials have been performed in end-stage cancer patients with high tumor loads. Here, in a phase I/II trial, we investigated the effect of autologous dendritic cell vaccination in 10 patients with acute myeloid leukemia (AML). The Wilms' tumor 1 protein (WT1), a nearly universal tumor antigen, was chosen as an immunotherapeutic target because of its established role in leukemogenesis and superior immunogenic characteristics. Two patients in partial remission after chemotherapy were brought into complete remission after intradermal administration of full-length *WT1* mRNA-electroporated dendritic cells. In these two patients and three other patients who were in complete remission, the AML-associated tumor marker returned to normal after dendritic cell vaccination, compatible with the induction of molecular remission. Clinical responses were correlated with vaccine-associated increases in WT1-specific $CD8^+$ T cell frequencies, as detected by peptide/HLA-A*0201 tetramer staining, and elevated levels of activated natural killer cells postvaccination. Furthermore, vaccinated patients showed increased levels of WT1-specific IFN-γ–producing $CD8^+$ T cells and features of general immune activation. These data support the further development of vaccination with *WT1* mRNA-loaded dendritic cells as a postremission treatment to prevent full relapse in AML patients.

cancer vaccine | active specific immunotherapy | phase I clinical trial

Overall, acute myeloid leukemia (AML) has a bad prognosis; indeed, only 23.8% of patients are alive 5 y after diagnosis (1). One of the main reasons for this poor outlook is that the majority of patients will relapse, despite reaching complete remission with classical polychemotherapy (2–4). Relapse is caused by the persistence of malignant cells, also designated as minimal residual disease, during complete remission. Furthermore, most AML patients are more than 60 y old, and the prognosis in this age group is worse because of comorbidity and higher rates of chemotherapy resistance (5).

One treatment with demonstrable effect on residual disease, relapse rate, and survival is allogeneic hematopoietic stem-cell transplantation, in which donor T lymphocytes exert a graft versus leukemia effect. However, allogeneic hematopoietic stem-cell transplantation is still beset by morbidity and mortality issues that preclude its routine use in older AML patients (3, 4). Thus, except for allogeneic hematopoietic stem-cell transplantation in subsets of younger AML patients, there is no consensus as to which postremission treatment should be applied to prevent relapse (3–6).

Recently, immunotherapeutic strategies have been developed to raise autologous antileukemic immunity to control malignant disease. Vaccination with peptides derived from leukemia-associated antigens has led to clinical and immunological responses in AML (7). These antigens include proteinase 3, from which the immunogenic PR1 peptide is derived (8–10), Wilms' tumor 1 (WT1) protein (8, 10–12), and the receptor for hyaluronic acid-mediated motility (RHAMM/CD168) (13). Although promising, the peptide-vaccination approach has limitations; in particular, the peptides need to be tailored to the MHC antigens of the patient, and not all MHC-restricted peptides are yet defined. This problem can be circumvented by introducing the full-length antigen into professional antigen-presenting cells, such as dendritic cells (DC), which will present multiple epitopes to T lymphocytes in the context of autologous MHC. We have developed a highly efficient, transient, and clinically safe transfection procedure to introduce mRNA encoding an antigen into DC by electroporation (14), a process that results in efficient antigen presentation (15). DC have been intensively investigated as cellular adjuvants for therapeutic cancer vaccination, and since the first reported trial in lymphoma patients in 1996 (16), DC have shown an unsurpassed capacity to induce in vivo antitumor responses. However, the overall clinical response rate, if any, remains very low (17). This poor clinical outcome could be, at least in part, ascribed to the fact that most clinical DC trials to date have been performed in end-stage cancer patients, often with bulky tumor loads and a compromised immune system caused by intensive treatment schedules and/or advanced disease stage (18). These observations plead for a more careful clinical-trial design that selects patients with residual but substantially reduced disease in whom immunotherapeutic interventions could significantly improve the clinical outcome after standard chemotherapy treatment (19). In lymphoma, this strategy used with patient-specific idiotype vaccination led to remarkable complete molecular responses (20). However, to our knowledge, such results have never been reported for DC-based cancer vaccines.

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Here, we present the results of a phase I/II vaccination trial using autologous monocyte-derived DC electroporated with *WT1* mRNA in AML patients who achieved complete or partial remission after polychemotherapy but remained at high risk of full relapse. *WT1* is overexpressed in the vast majority of AML cases (21–25). Furthermore, there is evidence that it plays an important role in the malignant phenotype of AML (24, 26–29). In an immunodeficient mouse model, there is a selective elimination of leukemic stem cells, but not normal human progenitors, and leukemia cells by WT1-specific cytotoxic $CD8^+$ T cells (30–32). Of note, in a prioritization study carried out by the National Cancer Institute, WT1 was selected from 75 defined tumor antigens to rank as the most promising cancer vaccine target (33).

The expression of *WT1* mRNA in bone marrow, or preferably, in peripheral blood, has been shown to be a relevant tumor marker in AML (25, 34–38). Especially after treatment, it has a high positive-predictive value as a molecular residual-disease marker (i.e., *WT1* mRNA expression levels above background in peripheral blood always herald clinical relapse) (25, 36, 37, 39). Moreover, failure to reduce WT1 transcripts below the threshold limits after chemotherapy invariably predicts relapse in patients with complete remission, which enables the early prediction of treatment outcome and the distinction of patients with continuous complete remission from those with only apparent complete remission (25, 39).

In this study, we show the immunogenic and antileukemic activity of a WT1-targeted DC vaccine in AML patients, evidenced by the conversion of partial to complete remission and the induction of molecular remission. Importantly, we found WT1-specific and nonspecific immunological correlates of these clinical responses.

Results

Clinical Results. The clinical details of the 10 AML patients recruited into this study are summarized in Table S1. Successful vaccine production was obtained in all patients from a single apheresis procedure (10–15 L), and DC vaccination was well-tolerated. In all patients, there was local erythema and induration at the site of injection, starting from the second vaccination. Patient with unique patient number (UPN)09 reported pain at the level of the draining axillary lymph nodes after DC vaccination. In patient UPN016, the platelet count dropped after the first DC injection and normalized 5 wk after the fourth vaccination (Fig. 1); she also experienced a mild flare-up of a preexisting inflammation of the Achilles and foot tendons, which started around the period of the fourth DC vaccination.

The most striking and demonstrable clinical effects were observed in patients UPN08 and UPN16, who were both in partial remission after chemotherapy and reached complete remission after DC vaccination. Before DC vaccination, these two patients were refractory to chemotherapy, and AML disease was not controlled according to classical morphological criteria [i.e., the percentage of myeloblasts in the bone marrow was increased above normal and was higher after the last (consolidation) chemotherapy than after the first (induction) chemotherapy]. After DC vaccination, the myeloblast percentage decreased to normal, from 6% to 1% in UPN08 (Fig. 1*A*) and from 9% to 0.3% in UPN16 (Fig. 1*B*). This was confirmed by flow-cytometric and histological examinations of the bone marrow. Concomitant with these changes, there was normalization of elevated *WT1* mRNA levels in peripheral blood after DC vaccination (Fig. 1). Patient UPN016 has relapsed in the bone marrow 9 mo after the start of DC vaccination, and this relapse was preceded by an increase in *WT1* mRNA expression levels above normal (Fig. 1*B*).

The return to normal values of the *WT1* tumor marker not only confirmed the antileukemic effect of DC vaccination in patients in partial remission, but it also revealed efficacy in some patients in complete remission (UPN01 and UPN06). In these latter subjects, *WT1* mRNA expression levels also illustrated the dynamics of minimal residual disease and the temporary nature

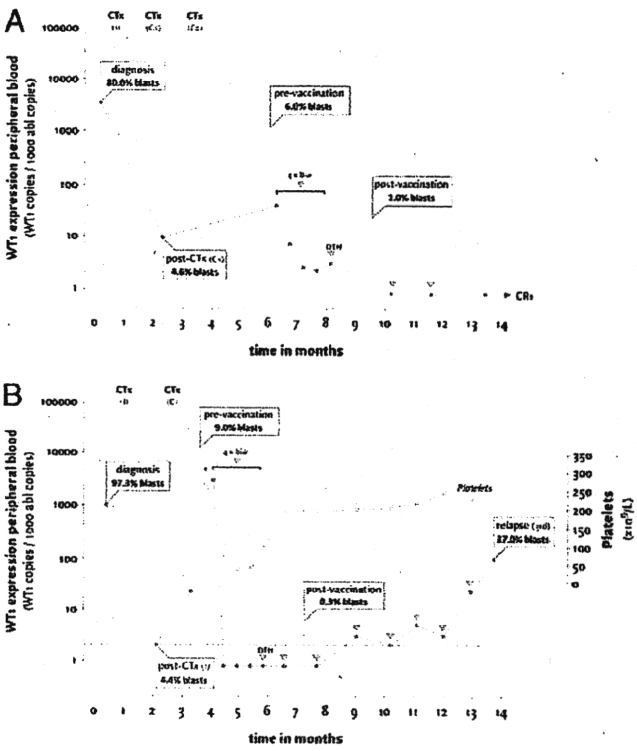

Fig. 1. Induction of complete remission by DC vaccination in patients UPN08 (*A*) and UPN16 (*B*). The gray-striped bars indicate the periods of chemotherapy (CTx) administration with subsequent hematological recovery from bone-marrow aplasia (I, induction chemotherapy; C, consolidation chemotherapy; C1, first cycle; C2, second cycle). A detailed description of the administered chemotherapeutic regimens is provided in Table S1. The brown arrowheads indicate the time points of DC immunization; the first cycle consisted of four biweekly (biw) injections (4 × biw) followed by DTH immunomonitoring testing. Both patients achieved a complete remission after four biweekly DC vaccinations, as evidenced by normalization of the myeloblast percentage in the bone marrow (inserts) and *WT1* mRNA expression levels (blue line). The horizontal dashed line represents the upper normal limit of *WT1* mRNA expression in peripheral blood. In patient UPN16, DC therapy was accompanied by a transient thrombocytopenia (dotted green line). Note the refractoriness to chemotherapy with abnormally increased bone-marrow blast cell percentage and *WT1* expression after the last chemotherapy course before DC vaccination. Patient UPN16 eventually relapsed in the bone marrow, and this relapse was preceded by molecular relapse as indicated by the loss of control of *WT1* expression levels.

of DC vaccine-induced control. After normalization of *WT1* mRNA expression associated with the initial round of DC vaccinations, this tumor marker increased on different occasions, compatible with molecular relapse. This was reversed by additional rounds of DC vaccination, which were administered usually on a bimonthly basis (Fig. 2). Patient UPN01 relapsed almost 4 y after starting DC vaccination, and elevated levels of *WT1* mRNA in peripheral blood were observed 3 mo before relapse. Thus, as in patient UPN16, molecular relapse preceded morphological and clinical relapse.

As of May 2010, three patients are in continuous complete remission with a normal peripheral blood picture. Of those three patients, two had increased *WT1* mRNA levels in blood that normalized post-DC vaccination; *WT1* expression normalized after the second DC vaccination in patient UPN08 (Fig. 1*A*) and after an additional fifth DC vaccine administered 2 mo after the initial cycle of four biweekly DC vaccinations (UPN06) (Fig. 2). In the third patient (UPN10), we observed a normalization of bone-marrow *WT1* mRNA expression from an increased value before DC vaccination to background levels postvaccination. This normalization

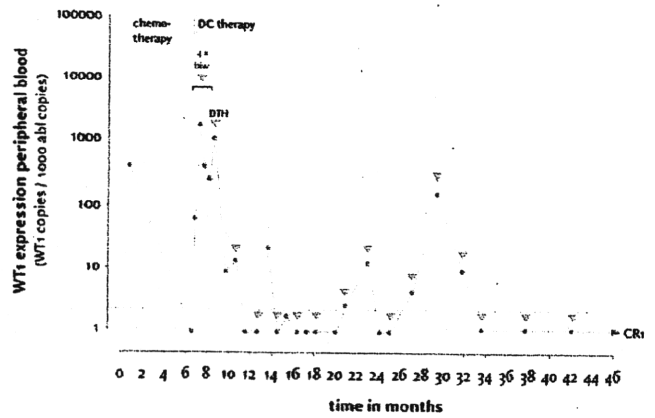

Fig. 2. Longitudinal control of AML minimal residual disease with repetitive DC vaccinations in patient UPN06. The gray-striped bar indicates the time period for induction and consolidation chemotherapy (CTx; details in Table S1) and subsequent recovery from bone-marrow aplasia. The brown arrowheads indicate the time points of DC vaccination; the first cycle consisted of four biweekly injections (4 x biw) followed by DTH immunomonitoring. The kinetics of *WT1* mRNA expression levels in peripheral blood are represented by the blue line, showing a normalization below the background threshold (horizontal dashed line) after chemotherapy and a first molecular relapse that was reversed with five successive DC vaccinations. Increases in *WT1* mRNA expression were observed on several occasions and were consistently controlled by maintenance DC vaccine administrations on a bimonthly basis. This graph is also representative of similar observations in patient UPN01, with the difference that patient UPN01 relapsed molecularly (i.e., loss of control of *WT1* mRNA expression levels) and subsequently, morphologically in the bone marrow.

of the *WT1* mRNA tumor marker seen after DC vaccination is indicative of molecular remission. In the two other patients who responded to DC vaccination but eventually relapsed, *WT1* mRNA expression in blood initially normalized after the first DC vaccination (UPN16) (Fig. 1*B*) and after the second DC vaccination (UPN01). As of now, 5 of 10 patients can be considered as clinical responders (UPN01, UPN06, UPN08, UPN10, and UPN16) and 3 of them as long-term responders (UPN01, UPN06, and UPN08), based on a complete remission status lasting for at least 3 y. Seven patients died, six because of relapse of AML, and the seventh had a progressively deteriorating clinical condition, presumably caused by adenocarcinoma of the lung (UPN07). In these seven patients, *WT1* mRNA expression either did not normalize postvaccination in blood (UPN03, UPN07, and UPN09) or bone marrow (UPN02 and UPN05) or returned to pathologically increased values after initial normalization (UPN01 and UPN16).

Immunomonitoring. Immunomonitoring was performed on peripheral blood mononuclear cells (PBMC) and plasma samples obtained before and after DC vaccination. Immunophenotypic analyses revealed no significant changes in the circulating frequencies or absolute numbers of lymphocyte subsets [$CD4^+$ and $CD8^+$ T cells, B cells, and natural killer (NK) cells]. Notably, DC vaccination did not affect the relative frequencies of regulatory $CD4^+$ T (Treg) cell subsets, including naturally occurring $CD25^+FoxP3^+$ Treg and induced IL-10^+ and/or TGF-β^+ Treg. Furthermore, the relative frequencies of naïve ($CD45RA^+CD62L^+$), terminally differentiated effector ($CD45RA^+CD62L^-$), effector memory ($CD45RA^-CD62L^-$), and central memory ($CD45RA^-CD62L^+$) subsets within the $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell compartments remained unchanged. However, a significant increase in circulating levels of plasma IL-2 and activated HLA-DR^+CD4^+ T cells was observed postvaccination, irrespective of clinical response (Fig. S1). More importantly, we found a significant correlation ($P = 0.01$) between clinical responses and the presence of high numbers of activated NK cells postvaccination (i.e., more than 40% HLA-DR^+ cells within the total NK cell population in four of five clinical responders and zero of five nonresponders). Furthermore, the level of HLA-DR^+ NK cells in all evaluable patients postvaccination was significantly higher compared with healthy volunteers [$32.25 \pm 16.8\%$ vs. $19.9 \pm 10.9\%$, respectively (mean $\pm$ SD; $n = 10$); $P = 0.04$], whereas the prevaccination levels were not significantly increased [$26.8 \pm 17.7\%$ vs. $19.9 \pm 10.9\%$ (mean $\pm$ SD; $n = 10$); $P = 0.25$].

WT1-specific immune responses were evaluated using anti-WT1 antibody analysis, WT1 peptide/HLA-A*0201 (pHLA-A*0201) tetramer staining, and functional intracellular cytokine assays. Analysis of humoral anti-WT1 responses showed relatively low, but not significantly different, concentrations of WT1 antibodies in the plasma before and after vaccination. Subclass analysis of WT1 antibodies showed a predominant T helper 1-associated IgG2 subtype.

Because patient accrual in our trial was independent of HLA haplotype caused by the polyepitope full-length antigen strategy, only five patients (UPN01, UPN05, UPN08, UPN09, and UPN16) were evaluable by pHLA-A*0201 tetramer analysis. Four pHLA-A*0201 tetramers specific for different HLA-A*0201-restricted WT1 epitopes were used: $WT1_{37-45}$, $WT1_{126-134}$, $WT1_{187-195}$, and $WT1_{235-243}$. Increased (>1.5-fold) frequencies of WT1-specific tetramer$^+CD8^+$ T cells (11) were observed in UPN01 and UPN08 compared with UPN05, UPN09, and UPN16 (Table 1). A significant positive correlation ($P = 0.025$) was found between long-term responders (UPN01 and UPN08) and an increase in WT1-specific tetramer$^+CD8^+$ T cells. Interestingly, this association was observed for more than one of the epitopes studied (i.e., $WT1_{187-195}$ and $WT1_{235-243}$ in UPN01 and all four epitopes examined in UPN08), suggesting a clinically relevant polyepitope-specific T cell response (Table 1). In patient UPN16, who responded to DC vaccination acutely but not in the long term, there was no increase postvaccination in the frequency of WT1-specific $CD8^+$ T cells, but the frequency of HLA-DR^+ NK cells nearly doubled compared with prevaccination levels (from 29% to 57%); this observation suggests that the short-term antileukemic effect seen in patient UPN16 was mediated, at least in part, by NK cells rather than by $CD8^+$ T cells.

Table 1. Overview of WT1-specific tetramer$^+CD8^+$ T cell frequencies pre- and post-DC vaccination in the peripheral blood of HLA-A*0201$^+$ AML patients

	Percent $WT1_{37-45}$		Percent $WT1_{126-134}$		Percent $WT1_{187-195}$		Percent $WT1_{235-243}$	
	Pre-DC	Post-DC	Pre-DC	Post-DC	Pre-DC	Post-DC	Pre-DC	Post-DC
UPN01*	0.016	0.002	0.006	0.006	0.006	0.011	0.004	0.009
UPN05	ND	ND	0.215	0.157	0.174	0.064	ND	ND
UPN08*	0.015	0.097	0.017	0.187	0.010	0.086	0.026	0.065
UPN09	0.078	0.003	0.102	0.002	0.036	0.004	0.036	0.005
UPN16	0.212	0.040	0.204	0.007	0.253	0.009	0.029	0.034

ND, not determined.
*Long-term responders.

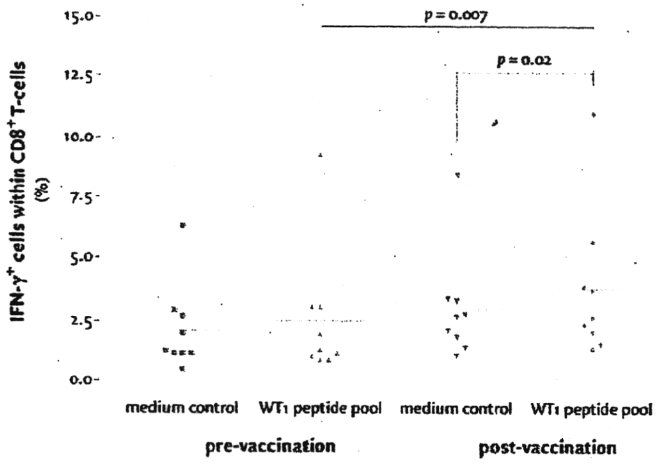

Fig. 3. Increase in WT1-specific IFN-γ–producing $CD8^+$ T cells postvaccination. PBMC were restimulated using *WT1* mRNA-electroporated mature DC for 1 wk. After 7 d, cultured PBMC were rechallenged using a WT1 peptide pool and assayed for intracellular IFN-γ production. Rechallenge with medium served as the negative control in all cases. Intracellular cytokine staining showed significantly higher percentages of IFN-γ–producing $CD8^+$ T cells postvaccination compared with antigen stimulation of PBMC obtained prevaccination (P = 0.007) and compared with a medium control (P = 0.02; n = 9; insufficient cell numbers were available from patient UPN05 for culture and subsequent analysis).

Detection of functional WT1-specific T cells was performed using a 1-wk in vitro antigen rechallenge protocol followed by intracellular cytokine staining. This approach revealed a significant increase in WT1-specific IFN-γ–producing $CD8^+$ T cells postvaccination (Fig. 3) compared with both prevaccination and medium controls. However, there was no correlation between antileukemic clinical responses and the frequency of WT1-specific IFN-γ^+CD8^+ T cells. Moreover, by assaying simultaneously for CD107a mobilization and intracellular IL-2, IFN-γ, TNF-α, and IL-10 production on a single-cell level after in vitro restimulation with *WT1* mRNA-electroporated or WT1 protein-loaded DC, polyfunctional WT1-specific T cells were not identified in any substantial numbers. Keyhole limpet hemocyanin (KLH)-specific T cell responses were readily detected in a 3-d in vitro restimulation assay, revealing a partially T helper 2 (Th2)-skewed cytokine profile characterized by secretion of IL-5, IL-10, and TNF-β.

In addition to these in vitro T cell responses, all patients exhibited in vivo delayed-type hypersensitivity (DTH) skin reactions (indurations of ≥2 mm) after four vaccinations to the vaccine (i.e., KLH-exposed *WT1* mRNA-electroporated DC) and to KLH separately as well as to nontransfected or *WT1* mRNA-transfected DC not exposed to KLH.

Discussion

In this phase I/II DC vaccination study, we have shown that *WT1* mRNA-electroporated autologous DC are immunogenic and that they induce a measurable antileukemic effect in AML patients treated with polychemotherapy but at high risk of full relapse. DC vaccination was associated with the achievement of molecular remission in 5 of 10 patients who exhibited elevated *WT1* mRNA expression levels before therapy. Moreover, in two of these patients, partial remission with demonstrable AML disease was converted into complete remission by the four biweekly DC vaccinations. Three of these five patients, including patient UPN08 who was in partial remission before vaccination, are currently alive and well with normal blood counts and normal blood *WT1* mRNA levels. It should be noted that all of the five responding patients were expected to experience a full-blown relapse based on partial remission status indicating morphologically demonstrable AML disease (3) and/or increased *WT1* mRNA levels after chemotherapy (25, 36, 37, 39). Of note, two of five responding patients eventually relapsed. These relapses were preceded by loss of control of *WT1* mRNA expression levels, confirming the value of this parameter as a predictive tumor marker for AML relapse. Loss of clinical and molecular response has also been described in WT1 peptide-vaccination studies (10–12).

The close temporal relationship between DC vaccination and the antileukemic effect is a strong argument for a causal association. More importantly, clinical responses were correlated with both innate (i.e., superior levels of activated NK cells in responders) and adaptive (i.e., increased WT1 tetramer$^+$CD8$^+$T cell frequencies in long-term responders only) immune responses postvaccination, indicating that the vaccine elicited clinically relevant and vaccine-specific immunity. To date, only a few DC vaccine studies have attempted to evaluate the nonspecific, yet potentially clinically relevant, NK cell response to immunization (40), but there is an increasing body of evidence that DC–NK interactions during the early phase of innate immunity can impact the quality and magnitude of the subsequent adaptive immune response (41). In our trial, it can be hypothesized that the effect of DC vaccination in clinical responders is, at least in part, mediated initially by a (nonspecific) innate immune response but that a subsequent adaptive WT1-specific T cell response is necessary to mediate a long-lasting (defined as a remission period of more than 3 y) (42) clinical benefit, as observed in UPN01 and UPN08. This hypothesis fits with the observation that patient UPN16, who showed a strong initial clinical response immediately after vaccination associated with extremely high levels of activated NK cells (57% of all NK cells) yet without any evidence of increased WT1-specific $CD8^+$ T cell immunity, relapsed 9 mo after the start of DC vaccination and succumbed 3 mo later. Moreover, we observed that WT1-specific $CD8^+$ T cell populations in these long-term clinical responders targeted multiple HLA-A*0201-restricted WT1 epitopes (43), providing in vivo human proof of concept that single-antigen mRNA-based loading of DC results in polyepitope-specific T cell responses. The ex vivo peripheral blood WT1-specific tetramer$^+$CD8$^+$ T cell populations identified in the current trial were small but clearly identifiable and are consistent with the data of a recent study showing a lower level of WT1-specific CD8+ T cells in blood compared with bone marrow (44).

Notably, we also consistently observed signs of general immune stimulation postvaccination in the whole patient population, regardless of clinical outcome, as evidenced by significant increases in plasma IL-2 levels and frequencies of circulating activated $CD4^+$ T cells. Notwithstanding the fact that these observations were based on direct ex vivo blood analyses, in vitro antigen rechallenge assays failed to reveal significant increases in either WT1- or KLH-specific IL-2–secreting $CD4^+$ T cells postvaccination. However, there was a significant increase in the peripheral blood postvaccination of WT1-specific IFN-γ^+CD8^+ T cells in the patient population as a whole, but this was not correlated with clinical benefit. This lack of correlation has been reported previously in a WT1 peptide-vaccine trial in cancer patients (11). Thus, whereas numerous reports already indicated that T cell responses after DC vaccination in humans can be more complex than initially expected (45–48), our findings underscore the need for more appropriate functional T cell assays that provide a more global view of the vaccine-specific T cell repertoire (e.g., by detection of transient CD137 expression on $CD8^+$ T cells on antigen (re) challenge, which is independent of cytokine profile) (49). Furthermore, our data reiterate the importance of a comprehensive immunomonitoring approach that includes disease-localized tissue sampling. The positive DTH reaction to the vaccine and its single antigenic components, indicative of an in vivo cell-mediated immune response, indirectly points to a migration of at least

part of the DC vaccine to the lymph nodes, where it presumably elicited antivaccine T cell responses. However, further investigation is warranted, because it is possible that the inclusion of KLH as a noncognate $CD4^+$ T helper antigen might have negatively influenced the immunological and clinical outcome of the WT1-targeted DC vaccination in some patients, which a partially Th2-skewed KLH-specific T cell response might suggest.

The antileukemic effect of the first round of four biweekly DC vaccinations was transient in some patients, and additional bimonthly DC administrations were needed to induce repeated normalizations of the *WT1* mRNA tumor marker. Similar booster effects of DC vaccinations have been observed in melanoma patients (45). The reason for the occasionally transient effect of DC vaccination, which is also observed in WT1 peptide-vaccine trials, may be the lack of cognate $CD4^+$ T cell stimulation, which is otherwise considered to be necessary for the maintenance of long-term $CD8^+$ T cell memory. The inefficient WT1-specific $CD4^+$ T cell stimulation postvaccination was most probably caused by cytoplasmic antigen expression after mRNA electroporation, leading to predominant MHC class I antigen presentation. One way to tackle this limitation and potentially improve the efficacy of DC vaccination is to introduce MHC class II-skewing signals into the mRNA construct [e.g., dendritic cell lysosome-associated membrane glycoprotein (DC-LAMP)] (50).

In contrast to peptide-vaccination studies, which have shown clinical activity in AML (9–13), DC vaccination in AML patients to date has been ineffective or inconclusive (51–53). The clinical effectiveness of our DC approach may be caused by several factors. First and similar to analyses conducted in WT1 peptide-vaccination studies (10–12), the use of the sensitive *WT1* mRNA residual disease marker revealed a clinical effect in some complete remission patients with increased *WT1* mRNA expression levels indicative of ongoing leukemic activity (54). Second, we were able to obtain DC from blood $CD14^+$ monocytes in all cases from AML patients in remission, in contrast to the generation of DC from primary AML cells, which was only successful in a minority of patients tested (53). Third, the choice of the WT1 antigen (33), rather than leukemic cell lysates or AML-derived DC (51–53), may also be advantageous, because WT1 is already recognized immunologically in AML patients (8, 55, 56); furthermore, WT1-targeted vaccine approaches have been successful in increasing the specific immune response in such a way that they can control AML, at least in some patients, as evidenced here and in other studies (10–12). Fourth, our unique clinical trial design (57), which is in stark contrast to current DC trials focusing on end-stage cancer patients, may have contributed to the demonstrable clinical effects observed (58).

In conclusion, vaccination with *WT1* mRNA-electroporated DC exhibits antileukemic activity in AML patients and elicits both innate and adaptive immune responses correlated with clinical benefit. These findings support the further development of vaccination with WT1-loaded DC as an immunotherapeutic strategy to prevent relapse in AML. Finally, WT1 is overexpressed in a majority of malignancies (24, 56), and WT1 peptide vaccination has led to clinical responses in patients with various solid tumors (11, 56). Thus, the promising data presented here suggest that *WT1* mRNA-electroporated autologous DC might serve as a platform model for the development of a universal cancer vaccine in the adjuvant setting.

Materials and Methods

Patients. After informed written consent was obtained, patients with AML (except acute promyelocytic leukemia) diagnosed according to World Health Organization (WHO) criteria were enrolled in a phase I/II trial approved by the Ethics Committee of Antwerp University Hospital (ClinicalTrials.gov number NCT00834002). All patients were in hematological remission after at least one prior antileukemic chemotherapeutic regimen and were not enrolled until 1 mo after polychemotherapy. Complete remission was defined by the absence of blasts in blood and by less than 5% blasts in marrow. Partial remission was defined as a ≥50% decrease in marrow blasts with normalization of blood counts. Inclusion criteria were the absence of a matched sibling donor for allogeneic hematopoietic stem cell transplantation (allo-HSCT) (if ≤60 y) and high risk of full-blown relapse as defined by (*i*) insufficient disease control as in partial remission status (more than 5% blasts in the bone marrow) (3), and/or (*ii*) *WT1* mRNA levels increased above background after chemotherapy (35, 39), and/or (*iii*) age ≥ 61 y (3–5), and/or (*iv*) previous relapse (3), and/or (*v*) hyperleukocytosis at presentation (white blood cell count > 20,000/μL) (2), and/or (*vi*) poor risk cytogenetic or molecular markers at presentation (2–4) (Table S1).

DC Vaccination. Clinical grade DC vaccines were prepared after leukapheresis of nonmobilized blood (Cobe Spectra) and immunomagnetic selection using CliniMACS (Miltenyi Biotec) (57). Monocyte-derived *WT1* mRNA-loaded DC vaccines were generated as described previously (57). DC vaccines were administered intradermally at biweekly intervals as previously reported (57).

Immunomonitoring. Detection and subtyping of anti-WT1 antibodies in pre- and postvaccination plasma samples were performed as previously described (59) (more details in *SI Materials and Methods*). Cytokine plasma levels were determined with the Th1/Th2 multiplex immunoassay (BenderMed Systems). Direct ex vivo analysis for lymphocyte subsets and activation markers was performed by flow cytometry using directly conjugated monoclonal antibodies (BD Biosciences). In vitro T cell restimulation assays and detection of antigen-specific cytokine responses were performed as described in *SI Materials and Methods*.

For detection of circulating WT1-specific $CD8^+$ T cells, pHLA-A*0201 tetramers were used. To minimize inter- and intrapatient variability, all tetramer analyses were performed on the same day by the same operator on a validated flow cytometer using identical reagents and instrument settings. Thawed peripheral blood mononuclear cells (PBMC) ($1–2 \times 10^6$ per experimental condition) obtained before vaccination and after the fourth vaccination were washed and stained for 15 min at 37 °C with phycoerythrin (PE)-labeled pHLA-A*0201 tetramers refolded with the following WT1 peptides: $WT1_{37–45}$, $WT1_{126–134}$, $WT1_{187–195}$, and $WT1_{235–243}$ (43). Cells were then washed and stained with the following mAbs: anti-CD8-Pacific Blue (Dako), anti-CD3-allophycocyanin (APC), anti-CD14-FITC, and anti-CD19-FITC (BD Biosciences). Dead cells were excluded using propidium-iodide staining (1 μg/mL; Sigma-Aldrich), and the FITC channel was used to exclude B cells and monocytes. For each sample, at least 40^4 viable $CD3^+CD8^+$ T cells within a standard lymphocyte gate were acquired using a CyFlow ML flow cytometer (Partec), and data were analyzed with FlowJo software (Tree Star). Because of the low frequencies of WT1-specific tetramer$^+$$CD8^+$ events detected in peripheral blood (Table 1), all tetramer data were reanalyzed in an independent and blinded fashion to ensure consistent interpretation as described previously (44).

To assess cell-mediated immunity in vivo, DTH skin testing was performed 2 wk after the fourth DC vaccination by intradermal injection, and measurement of induration was 48 h later.

Molecular Tumor-Marker Monitoring. Longitudinal tumor-marker monitoring in blood and bone marrow was performed by qRT-PCR for *WT1* gene expression as described previously (25, 35, 39). Values above 2 and 25 copies of *WT1* mRNA per 1,000 *ABL* copies in blood and marrow, respectively, were considered to be above normal background and thus, indicative of residual disease. More details are in *SI Materials and Methods*.

Data Mining and Statistical Analysis. Flow-cytometric data analysis was performed using FlowJo version 8.4.4 (TreeStar). GraphPad Prism 4.0 software (GraphPad Software) was used for graphical data representations and statistical computations. Statistical analysis was performed using Student *t* test or one-way ANOVA, where appropriate. Correlations between immunological and clinical responses were examined with a two-sided χ^2 test. Any *P* value <0.05 was considered statistically significant.

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CASE REPORT

WT1 peptide vaccine induces reduction in minimal residual disease in an Imatinib-treated CML patient

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Abstract

How to treat CML patients who are resistant to inhibitors of BCR-ABL tyrosine kinase such as Imatinib is a very important and urgent issue in clinical hematology. Here, we report a case of Imatinib-treated CML in which intradermally administered WT1 peptide vaccine elicited WT1-specific immune responses and the resultant reduction in the persistent residual disease in co-administration of Imatinib. BCR-ABL mRNA levels were being maintained under the detection limit for 8 months since week 77 of vaccination. No adverse effects except local erythema at the injection sites were observed. The tetramer assay revealed that the decrease in BCR-ABL mRNA levels was associated with the increase in frequency of WT1-specific cytotoxic T lymphocytes, notably effector-memory type of that, in the patient's peripheral blood. The case presented here indicates that WT1 peptide vaccine may become a safe and cure-oriented therapy for CML patients who have residual disease regardless of the treatment with Imatinib.

Key words WT1 peptide vaccine; CML; immunotherapy; Imatinib

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Accumulating evidence has shown that Imatinib does not eliminate leukemia cells in the most of chronic myelogenous leukemia (CML) patients despite its high effectiveness in the reduction in CML cells (1, 2). Here, we present a case of Imatinib-treated CML in which WT1-peptide vaccine elicited WT1-specific immune responses and the resultant reduction in persistent residual disease.

Case report

A 76-yr-old woman was diagnosed as having chronic-phase CML in April 2005. After treatment with Imatinib, she achieved a complete cytogenetic response (CCyR) in March 2006. However, major BCR-ABL mRNA was persistently detected in peripheral blood (PB) for 12 months after the achievement of CCyR. She therefore desired to be treated with WT1 peptide vaccine in combination with Imatinib. She had HLA-A*2402 and met the inclusion criteria for the phase I/II clinical study of WT1 peptide-based immunotherapy combined with Imatinib for chronic myelogeneous leukemia (#UMIN000003508, http://www.umin.ac.jp/english/). In addition to the continued administration of Imatinib, an intradermal injection of 1 mg of 9-mer modified WT1 peptide (p235-243:CYTWNQMNL) (3) emulsified with Montanide ISA51 adjuvant was begun on August 14, 2007, and repeated at 2-wk intervals in co-administration of Imatinib. The levels of major BCR-ABL mRNA in PB decreased to the detection limit at week 13. However, BCR-ABL mRNA levels gradually increased in associa-

tion with weakening of delayed-type hypersensitivity (DTH) to WT1 peptide and reached the maximum at week 38. As these results suggested the reduction in WT1-specific cytotoxic T lymphocyte (CTL) responses because of antigen-induced cell death of the CTLs, intervals of WT1 vaccination were extended from 2 to 3 or 4 wk. Following the extension, major BCR-ABL mRNA levels decreased again and were being maintained under the detection limit for 8 months since week 77 (Fig. 1). No adverse effects except local erythema at the injection sites were observed.

DTH reaction to WT1 peptide became positive after four WT1 vaccinations. The frequency of WT1-specific $CD8^+$ T cells (WT1 $tetramer^+$ $CD8^+$ T cells) among $CD8^+$ T cells in PB increased from 0.06% to 0.16% by the beginning of WT1 vaccination but declined to 0.07% in association with the weakening of DTH to WT1 peptide from 8×4 to 2×1 mm at week 35. However, the frequency of WT1-specific $CD8^+$ T cells increased from 0.07% to 0.23% again following the extension of vaccination intervals. It should be noted that after the WT1 vaccination, the frequency of effector-memory type of WT1-specific $CD8^+$ T cells, which were considered to be able to differentiate into effector cells and be mounted for cytotoxic activities upon antigen stimulation, markedly increased from 4.5% to 32.6% at week 5 and was kept in more than 30% of the WT1-specific $CD8^+$ T cells (Fig. 1).

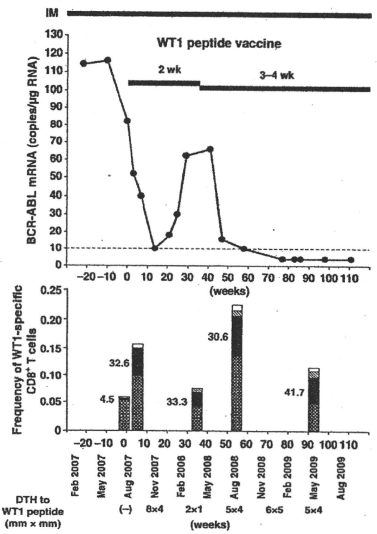

Figure 1 Clinical course of a CML patient who was vaccinated with WT1 peptide. Upper panel, reduction in minimal residual CML cells by WT1 peptide vaccine. WT1 peptide vaccine was intradermally administered initially at 2-wk and later 3–4-wk intervals. Minimal residual disease in the CML patient was detected by the measurement of major BCR-ABL mRNA in peripheral blood. Imatinib was daily administered at the dose of 200 mg because of her intolerance to Imatinib at higher doses. IM, Imatinib. Lower panel, WT1-specific immune responses. WT1-$tetramer^+$ $CD8^+$ T cells, which were negative for lineage markers such as CD4, CD14, CD16, CD19, and CD56, were considered to represent WT1-specific $CD8^+$ T cells. The frequency of WT1-specific $CD8^+$ T cells among the $CD8^+$ T cells was measured by flowcytometry. The differentiation status of WT1 $tetramer^+$ $CD8^+$ T cells was analyzed based on CD45RA and CCR7 expression as previously described (8). □, Naïve; ▨, central memory; ■, effector memory; and ▩, effector. Numbers on left sides of columns show the percentages of E-M type among WT1-specific $CD8^+$ T cells. Delayed-type hypersensitivity was measured 48 h after injection of WT1 peptide solution (10 μg/50 μL) without Montanide ISA51 adjuvant.

Discussion

Since stem/progenitor cells of CML cells that played an essential role in relapse and progression of the disease overexpressed the *WT1* gene (4, 5), they could be targeted by WT1-specific CTLs. Therefore, it is reasonable to think that WT1-specific CTLs induced by WT1 peptide vaccine eradicated the residual leukemogenic stem/progenitor cells and induced the reduction in BCR-ABL mRNA which reflected the minimal residual disease in this CML patient.

One important concern about TKIs is their possible mutagenic effects. In fact, although rare, emergence of abnormal Ph-negative clones after the treatment with Imatinib has been reported (6). These results indicate that TKI may have adverse effects on the incidence of secondary myelodysplastic syndrome (MDS) or leukemia (7). Therefore, combination of WT1 peptide vaccine with Imatinib could reduce dose of Imatinib and shorten treatment duration and might contribute to reduction in the risk of Imatinib-induced MDS/leukemia.

Taken together, the results presented here should indicate that WT1 peptide vaccine induced and/or enhanced WT1-specific immune responses, leading to induction of a clinical response. Therefore, the case presented here indicates that WT1 peptide vaccine may become a safe and cure-oriented therapy for CML patients who have residual disease regardless of the treatment with Imatinib. This conclusion should be confirmed by further studies with larger number of patients.

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Conflict of interest

The authors declare no conflict of interest.

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CRITICAL REVIEW

STEREOTACTIC RADIOTHERAPY OF PRIMARY LUNG CANCER AND OTHER TARGETS: RESULTS OF CONSULTANT MEETING OF THE INTERNATIONAL ATOMIC ENERGY AGENCY

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To evaluate the current status of stereotactic body radiotherapy (SBRT) and identify both advantages and disadvantages of its use in developing countries, a meeting composed of consultants of the International Atomic Energy Agency was held in Vienna in November 2006. Owing to continuous developments in the field, the meeting was extended by subsequent discussions and correspondence (2007–2010), which led to the summary presented here. The advantages and disadvantages of SBRT expected to be encountered in developing countries were identified. The definitions, typical treatment courses, and clinical results were presented. Thereafter, minimal methodology/technology requirements for SBRT were evaluated. Finally, characteristics of SBRT for developing countries were recommended. Patients for SBRT should be carefully selected, because single high-dose radiotherapy may cause serious complications in some serial organs at risk. Clinical experiences have been reported in some populations of lung cancer, lung oligometastases, liver cancer, pancreas cancer, and kidney cancer. Despite the disadvantages expected to be experienced in developing countries, SBRT using fewer fractions may be useful in selected patients with various extracranial cancers with favorable outcome and low toxicity. © 2011 Elsevier Inc.

Stereotactic body radiation therapy, Non–small-cell lung cancer, Lung metastases, Liver cancer, Pancreatic cancer, Kidney cancer.

INTRODUCTION

Cancer is one of the major health concerns worldwide. The burden of cancer is increasing globally, with 20 million new cases expected per year in 2020, half of which will be in developing countries (1). The inability to cope with the growing economic and societal burden of cancer is emblematic of the tremendous health disparities reflected in developing countries, which have only 5% of the global resources spent on cancer (2–3).

The proportion of cancer patients in developing countries requesting radiotherapy (RT) is likely higher than in regions of high income because of the types of cancers and the stages at which these tumors are diagnosed (4). Moreover, patients in developing countries are dealing with some issues that are not common in the developed world. They include patient transportation to the facility (5), social support, accessible local housing, and noncompliance with treatment. It was shown (6) that the use of short courses in selected patients could be cost effective and convenient, especially for patients coming from remote areas.

Although many countries have not yet established RT service, others have aging RT services, which are usually restricted to a few centers, mainly concentrated in large urban areas. RT is affordable for developing countries with large populations, but some regions with small populations have not invested in RT (7–10). Emerging new technologies for cancer treatment, however, are spreading widely, in both developed and developing countries. One of these, stereotactic body radiotherapy (SBRT), has been increasingly used in recent decades.

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The International Atomic Energy Agency (IAEA) has a crucial role in both developing new RT facilities and upgrading existing facilities, including equipment and human resources in developing countries. By organizing meetings of experts, the IAEA gathers advice in RT to establish RT facilities in member states. With such an aim, a meeting of consultants was held in Vienna in November 2006 to advise the IAEA on the state of the art of the use of SBRT in primary lung tumors and other body tumors. This article represents a summary of that meeting and subsequent (2007–2010) communication between experts on the recent developments in SBRT deemed necessary because of the fast developments in the field.

Stereotactic radiation characteristics

Characteristics attractive to developing nations. Several characteristics would make SBRT attractive to developing nations. Shortened treatment time with fewer fractions than usually used in developing countries would be a major consideration. This in turn would generally enable improved access to RT treatments in departments worldwide. In addition, shorter treatment (outpatient or inpatient) would also be more cost effective for both patients and hospitals. This would be realized by lessening travel from prolonged distances to and from hospitals, and secondly for hospitals having limited inpatient capabilities. Other attractive characteristics would include improved overall results such as local control, overall survival, and disease-specific survival. Lower toxicity, in addition, would also be an important issue from the standpoint of both better quality of life and less costly symptomatic care (including frequent hospitalization in a patient population having a notorious record of having excessive comorbidities) needed in such cases.

Obvious barriers to implementation in developing nations. There may be several barriers for successful implementation of SBRT of lung cancer in developing countries. They can be broadly separated into pretreatment and treatment issues, including low incidence in certain regions such as sub-Saharan Africa. The lack of modern and comprehensive diagnostic tools, such as computed tomography (CT) and positron emission tomography, would largely jeopardize appropriate diagnosis and staging of potential candidates. In addition, the vast majority of patients would fall into a locally advanced or metastatic category because of a lack of screening and early detection programs that may result in identification of suitable cases, *i.e.,* those having Stage I non–small-cell lung cancer. Of treatment-dependent obstacles, capital costs for obtaining an immobilization system would be the major issue, assuming that existing external-beam RT machines (primarily linear accelerators) have been properly maintained. Lack of previous exposure and experience with three-dimensional RT, seen as the logical parent of SBRT, may be an important obstacle. Barriers to successful implementation of SBRT also include insufficient staffing, inadequate training of personnel, and lack of a dedicated team for introducing and implementing this technique.

CURRENT STATUS OF SBRT DELIVERY IN THE DEVELOPED WORLD

Historical aspects and early experience

Intracranial stereotactic radiosurgery (SRS) was a novel treatment method when introduced in the middle of the 20th century, with conceptual parallels to brachytherapy in regard to the tight spatiotemporal distribution in dose delivery. The clinical experience with intracranial SRS, together with the technical developments in conventional RT, initiated the development of SBRT characterized by a very high dose per fraction, delivered in a short time. This was started at the Swedish Karolinska University hospital in 1991 with tumors in the liver and lungs (11, 12). In parallel the method was developed in Japan and clinically introduced in 1994 for lung tumors. During the last 5 years of the 1990s, SBRT was introduced in several centers in Europe, Japan, and the United States (13–19). The early reports had already shown very promising results with regard to local control and toxicity for the hypofractionation schedules that were adopted, with 10 to 15 Gy per fraction given in a few fractions during a short time (15, 20). However, owing to the new aspects introduced in SBRT, clinical experience was initially gathered at a very slow rate, and it was only during the past decade that outcome data from several centers were available to confirm the initial promising results.

Experience in primary lung tumors

Many studies with SBRT were conducted around the world in treating both primary and metastatic cancers within the lungs because of their high prevalence, the high rates of cancer-associated deaths, and the desire for more effective treatments. The experience in treating primary lung cancer using SBRT has been obtained mainly in patients unfit for surgical resection (*i.e.,* medically inoperable patients). Furthermore, nearly all reports described outcomes in patients with Stage I disease, particularly for peripheral tumor locations. Inasmuch as medically inoperable lung cancer patients are at risk for death of other causes, survival in these patients is ultimately compromised. Still, the benefits of SBRT were demonstrated by dramatically improved rates of local control.

Local tumor response. The local control rates of primary lung cancer with SBRT have been previously reported by several authors (Table 1): 94 % (47/50) for 50 to 60 Gy in five fractions with a median follow-up time of 36 months (21, 23); 92 % (22/24) for 60 Gy in eight fractions with a median follow-up time of 24 months (22, 24) 87 % (30/37) for 60 Gy in three fractions with a median follow-up time of 15 months (19); 85% for 48 to 60 Gy in eight fractions with a median follow-up time of 17 months (25); 95% for 45 to 56.2 Gy in three fractions with a median follow-up time of 10 months (26); 90% for 30 to 40 Gy in

Table 1. Local control rates of stereotactic radiotherapy for primary lung cancer

Study	Total dose (Gy)	Daily dose (Gy)	Reference point	Local control	Median follow-up time
Uematsu *et al.*, 2001 (21, 23)	50–60	10	80% margin	94%(47/50)	36 months
Arimoto *et al.*, 1998 (24)	60	7.5	Isocenter	92%(22/24)	24 months
Timmerman *et al.*, 2003 (19)	60	20	80% margin	87%(30/37)	15 months
Onimaru *et al.*, 2003 (25)	48–60	6–7.5	Isocenter	80%(20/25)	17 months
Wulf *et al.*, 2004 (26)	45–56.2	15–15.4	80% margin	95%(19/20)	10 months
Nagata *et al.*, 2005 (28)	48	12	Isocenter	97%(44/45)	30 months
Lee *et al.*, 2003 (27)	30–40	10	90% margin	90%(8/9)	21 months
Fakiris *et al.*, 2009 (29)	60–66	20–23	80% margin	88%(70)	50 months
Baumann *et al.*, 2009 (30)	45	15	67% margin	92%(57)	35 months
Timmerman *et al.*, 2010 (31)	60	20	80% margin	98%(54/55)	36 months

four fractions with a median follow-up time of 21 months (27); 97% (44/45) for 48 Gy in four fractions with a median follow-up time of 22-30 months (28); 88% for 60 to 66 Gy in three fractions with a median follow-up time of 50 months (29); and 92% for 45 Gy in three fractions with a median follow-up time of 35 months (30). The Radiation Therapy Oncology Group (RTOG) 0236 demonstrated a very good 3-year local control rate that was as high as 98% (31). Even though the definition of local control is different between each trial, a biologic effective dose (BED) larger than 100 Gy may be effective for SBRT of solitary lung cancers with a local control rate above 85%.

Survival. In a series of Stage IA disease (T1N0M0), the 1-year and 5-year local relapse-free survival rates were 100% and 95%. The disease-free survival rates after 1, 3, and 5 years were 80%, 72%, and 72%, respectively, and the overall survival rates were 93%, 83%, and 83%, respectively. In the Stage IB (T2N0M0) series of Nagata *et al.* (28), local relapse-free survival rates were 100%. The disease-free survival after 1, 3, and 5 years were 92%, 71%, and 71%, respectively, and the overall survival rates were 82%, 72$, and 72%, respectively. Onishi *et al.* (32) reported the results for 13 institutions in Japan, which summarized 245 patients: 155 with Stage IA lung cancer and 90 with Stage IB lung cancer. There were 87 operable and 158 inoperable patients, and their results showed that the intercurrent death rate was especially high in the inoperable patient group. Moreover, the 5-year survival rates of operable patients irradiated with more than BED = 100 Gy were 90% for Stage IA and 84% for Stage IB disease, and their clinical results were as good as those obtained by surgery.

Toxicities. The great concern of pulmonary toxicity with SBRT treatment was moderated by the very low rates of complications in early studies. Most pulmonary complications are less than Grade 2 according to the National Cancer Institute Common Terminology Criteria version 2.0. It is not uncommon for patients to experience rib fracture or chest wall pain months after SBRT, especially if tumors adjacent to the chest wall have been treated. Some of these patients, but not all, will have pleural effusions associated with the chest wall pain. The problem seems mostly to be self limited, and conservative management with over-the-counter analgesics or anti-inflammatory medicines is typically effective.

However, a few serious complications have recently been reported by several institutions in Japan (33). These include Grade 5 pulmonary complications, radiation pneumonitis, hemoptysis, and radiation esophagitis. Most cases of Grade 5 radiation pneumonitis were accompanied with interstitial pneumonitis.

Another concern of toxicity was the effects on the central bronchus, pulmonary artery, esophagus, heart, and spinal cord, for which a hypofractionated dose had not been followed up for a sufficiently long time. Lethal pulmonary bleeding and esophageal ulcer have been previously reported (33). Timmerman *et al.* also reported a series of complications with SBRT (34). Chang *et al.* reported on safely treating central tumors considering dose constraints with the SBRT technique (35). Nonetheless, central tumors adjacent to mediastinal organs should be carefully considered (36). Toxicities as reported in several articles are shown in Table 2.

The most important issue is to maintain the dose constraints of organs at risk (OAR) to avoid serious complications. The dose constraints of the OAR, including the spinal cord, pulmonary artery, bronchus, and heart under the Japan Clinical Oncology Group (JCOG) 0403 protocol, are shown in Table 3. The RTOG has enacted normal tissue

Table 2. Clinical toxicities after stereotactic radiotherapy for primary lung cancer

Study	Number of cases	Lung ≥Grade 3	Lung Grade 5	Other Grade 5
Uematsu *et al.*, 2001 (23)	50	0%	0%	
Arimoto *et al.*, 1998 (24)	24	NA	0%	
Lee *et al.*, 2003 (27)	28	0	0%	
Onimaru *et al.*, 2003 (25)	45	2%	0%	Esophagus
Wulf *et al.*, 2004 (26)	61	0	0%	
Nagata *et al.*, 2005 (28)	45	0	0	
Timmerman *et al.*, 2006 (34)	70	20%	9%	Hemoptysis, pericarditis
J-CERG, 2009 (33)	2,106	NA	0.6%	Esophagus, hemoptysis

Table 3. Dose and volume constraints for organs at risk in stereotactic body radiotherapy of lung tumors according to Japan Clinical Oncology Group 0403

Organ	Dose	Volume	Dose	Volume
Lung	40 Gy	≤100 cc	MLD	≤18 cc
	V_{15}	≤25%	V_{20}	≤20%
Spinal cord	25 Gy	Maximum		
Esophagus	40 Gy	≤1 cc	35 Gy	≤10 cc
Pulmonary artery	40 Gy	≤1 cc	35 Gy	≤10 cc
Stomach	36 Gy	≤10 cc	30 Gy	≤100 cc
Intestine	36 Gy	≤10 cc	30 Gy	≤100 cc
Trachea, main bronchus	40 Gy	≤10 cc		
Other organs	48 Gy	≤1 cc	40 Gy	≤10 cc

Abbreviation: MLD = Mean Lung Dose, Other organs do not include chest wall & liver.

constraints for RTOG 0618 treating operable patients with early-stage primary lung cancer (Table 4).

Clinical trials. Prospective Phase II testing of SBRT in operable patients is currently ongoing in Japan (JCOG 0403) and the United States (RTOG protocol 0618). In medically inoperable patient groups, a Nordic multi-institutional consortium is comparing three-fraction SBRT to conventional RT in an ongoing randomized Phase II study. The RTOG has finished a Phase II study of three-fraction SBRT for peripheral tumors and is planning a Phase I study with five fractions in patients with central tumors. Finally, the JCOG is finishing a Phase II study using a four-fraction treatment for peripheral tumors and is starting a Phase II study using a higher dose specifically for T2 tumors as JCOG 0701.

Experience in metastatic lung tumors

The experience in treating lung metastasis has been mostly with oligometastases. In contrast to patients with primary lung cancer, patients with metastases do not inherently have poor pulmonary function secondary to tobacco abuse. As such, the toxic effects of treatment would not be expected to be identical between these differing populations. In addition, there is increasing evidence that it may be more difficult to attain local control in metastatic tumors than in primary lung cancer. This would argue for a higher treatment dose (controlled for tumor volume) for metastatic tumors than for primary presentations. Unfortunately, the results of treating lung metastases were frequently included in the reports of patients treated with primary lung cancers, making interpretations of the results more difficult (20, 37–39). Recently a few articles were published that focused on lung metastases (40, 41). Still, SBRT has a relatively high rate of local control per lesion, making it an effective treatment for selected patients with oligometastases.

Table 4. Dose constraints for normal tissue related to steepness of dose gradients from target according to Radiation Therapy Oncology Group 0618 for stereotactic body radiotherapy in operable patients with lung cancer

Organ	Volume	Dose (cGy)
Spinal cord	Any point	18 Gy (6 Gy/fraction)
Esophagus	Any point	27 Gy (9 Gy/fraction)
Ipsilateral brachial plexus	Any point	24 Gy (8 Gy/fraction)
Heart/pericardium	Any point	30 Gy (10 Gy/fraction)
Trachea and ipsilateral bronchus	Any point	30 Gy (10 Gy/fraction)
Whole lung (right & left)	V_{20}	Less than 5–10% of total lung volume
Skin	Any point	24 Gy (8 Gy per fraction)

Experience in liver tumors

Treatment of liver tumors is the second highest indicator for SBRT. Surgical data have shown that local treatment of liver tumors—mostly hepatocellular carcinoma and metastases—can be curative in up to 25–30% of patients if patient selection is appropriate (42). Nevertheless a significant proportion of patients will not be suitable for surgery because of age, medical comorbidity, or intrahepatic localization of the tumor (bilobar, adjacent to large vessels/portal structures). For these cases, SBRT is completely noninvasive and compares favorably with actuarial local control rates of at least 80% after 2 years (16, 20, 43, 44). Acute toxicity is mild. Clinically relevant subacute or late toxicities are not reported, if OAR have been kept out of the high dose area. Nevertheless, local control is dependent on dose, with recurrences occurring even after years, (*e.g.,* with single doses below 26 Gy/isocenter or 3 × 10 Gy/planning target volume [PTV] enclosing 65% isodose) (45, 46). By contrast, some authors have shown that significantly higher doses can be applied safely, such as single doses above 30 Gy/isocenter or 3 × 20 Gy/PTV enclosing 80% isodose, if the normal tissue dose constraints are respected (46–49).

Experience in retroperitoneal (pancreas and kidney) tumors

Abdominal retroperitoneal tumors pose a difficult challenge in view of their proximity to the poorly tolerant bowel. In the case of pancreas tumors, trials have shown conflicting results about the benefit of therapy. Although Hoyer *et al.* indicated little benefit and increased toxicity in patients treated with 45 Gy in three fractions (50), Koong *et al.* used a single dose ranging from 15 to 25 Gy and were able to control tumors in most patients with acceptable toxicity (51, 52).

Although renal cancers are thought to be radioresistant when treated with conventional fractionation schedules, Wersaell *et al.* found extremely high rates of local control with a three- to four-fraction SBRT regimen (53). These results concurred with the high local control rates observed when SRS with a large dose per fraction was used to treat brain metastases of the same histology.

Biology of dose delivery to tumor and normal tissues

Unlike normofractionated RT, the biologic purpose of SRT is for lethal rather than sublethal cell damage in the

high-dose area without repair. Additionally, because of the short overall treatment time (single dose, hypofractionation within 1 to 3 weeks), avoiding the repopulation of tumor cells is another advantage. On the other hand, the presumption is that reoxygenation and redistribution of cells in the cell cycle will not occur with the prescribed dose. The OAR are prevented from serious damage by sparing these tissues from the high-dose area.

Besides dose escalation trials for lung and liver tumors (47, 49), prospective institutional-based reports on the clinical results of SBRT have been published. Unfortunately, comparison of these results is difficult because different dose fractionation schedules have been used, and there is lack of uniformity in normalization and prescribed doses. To overcome this problem, some authors used the BED based on the formula BED (Gy) = dose/fraction × fraction number (1 + fraction dose / α/β using an α/β of 10 Gy for tumor tissue (54, 55). They found a BED of about 100 Gy to be appropriate to achieve a tumor control probability of about 90% for lung tumors. Because it has not been proved that the LQ (linear quadratic) model will be reliable at such high fraction doses, other radiobiologic models might be better suited to predict the effect of SBRT, including modifications of the multitarget model (56).

MINIMUM METHODOLOGY/TECHNOLOGY REQUIREMENTS

Imaging for planning

Imaging for treatment planning is usually based on CT data, whereas magnetic resonance imaging or positron emission tomography can assist this purpose. Before definite scanning, potential breathing mobility has to be evaluated. Depending on the method used to decrease breathing mobility, the amount of motion should be analyzed (it has to be performed to determine the appropriate margins for PTV definition). This can be done by either four-dimensional CT, multislice CT, dynamic scans (repeated scans at the same couch position), or evaluation of the target position during maximum inspiration and expiration. Although this approach is based on slices, which show the scanned tumor position in a very short (<1 second) time, resulting in a sharp image, the target can also be scanned by slow CT. With this technique the tumor is scanned very slowly (*e.g.*, scan time for a slice of 3 seconds). The image shows a blurred shape of the target, including and depending on internal motion (57), which represents the orbit of the moving target. This technique might have advantages, especially when cone-beam CT is used for target verification before irradiation, because the slow scan time (about 1 minute) will cause the shape of the target to also seem blurred (58).

Planning processes

Clinical experience from SBRT has indicated that geometric errors (of a magnitude that is not too uncommon in RT) may lead to more severe consequences than errors in dose delivery. Thus, if priorities need to be determined, geometric aspects should be emphasized more than dose aspects in the planning and delivery processes of SBRT.

Treatment planning in SBRT is done on commercial treatment planning systems, which are also used for RT planning in general. The CT data must account for the different densities in the body for the dose calculation. For dose calculation of tumors in the lungs, pencil beam algorithms have a limited accuracy but are acceptable for use (59). Point kernel–based superposition/convolution algorithms give a more accurate estimate of the dose to the tumor and surrounding lung tissue (60). The error in the dose calculation for tumors in the lungs is reduced if the photon energy is restricted a maximum of 6 MV. Small field sizes are often used in SBRT because of the small size of the PTV. Thus, accurate beam modeling is important (both profiles and depth doses) for field sizes down to 3 cm × 3 cm, preferably down to 2 cm × 2 cm. Image registration tools for the geometric verification process, dose–volume histogram calculation tools, and tools (for example rulers) to calculate the position of the isocenter in the reference system defined by the fiducials must be included.

Radiation beam delivery equipment

Clinical experience with SBRT stems primarily from the use of conventional linear accelerators, and to a lesser extent from more specialized accelerators, but not from the use of conventional cobalt units. The latter is not recommended for SBRT because of the lack of clinical experience and the inferior physical characteristics of the beams.

The following recommendations are given for the linear accelerator for SBRT: Photon energies of 6 MV (or close to that) for tumors in the lungs. For tumors below the diaphragm (not passing through lung tissue), 6 to 20 MV. It is important to keep the treatment time reasonably short, preferably in the range of half an hour per target, as a maximum. The reason is mainly to avoid geometric errors from patient motion during a very long treatment time, but also to some extent to avoid a possible dose–rate effect. The following aspects are related to a short treatment time. A multileaf collimator (leaf width maximum 1 cm) should preferably be used to shape the beams, but customized blocks may be acceptable. The preferred dose rate should be at least 400 MU/min, but at least 250 MU/min can be acceptable Motorized wedges should preferably be used, but manual wedges may be acceptable. The size of the mechanical isocenter sphere should be within 1 mm in radius. Equipment (*e.g.*, lasers, video cameras, X-ray sources) in the treatment room used for setup should be accurately adjusted to the isocenter. The deviation of the actual isocenter point from the planned one should be aimed to be within 1 mm in the reference system defined by the fiducials (Figure). The mechanical sag on the treatment couch with the patient in treatment position and CT couch must be checked, and should be of the same order. This is of primary importance for targets extended in the cranial-caudal direction.

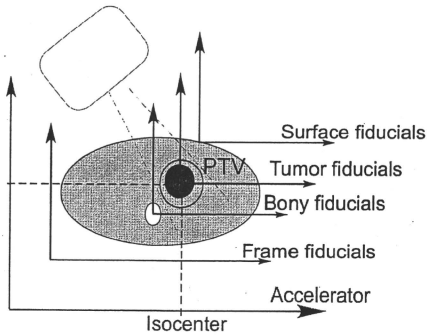

Fig. Patient treatment in the stereotactic body frame (ELEKTA Instr.). The correct isocenter position of the target and adequate suppression of breathing mobility by increased abdominal pressure is proved on the treatment couch by a mobile computed tomography device with gantry movements (Tomoscan M, Philips, Inc.).

Immobilization

Immobilization of the patient should be comfortable and also rigid to avoid intrafractional motions to ensure accurate repositioning of the patient between treatment planning and irradiation sessions. Both goals are usually achieved by tightly moulded vacuum pillows, which are attached to a stereotactic frame (e.g. SBF) or are used frameless (*e.g.*, Body-Fix). Specific attention should be directed to providing comfortable support for the arms and legs/knees, because they are most prone to become uncomfortable during a long treatment procedure (verification and irradiation might last up to 60 min).

Geometric verification

Geometric verification is a very important issue for SBRT because its single dose is usually more than 10 Gy and therefore constitutes 20–33% of the whole dose. A single misalignment will result in local failure or severe complication. The most simple verification method widely accepted is an anterior–posterior portal film taken before each session to compare with DRR (Digitally reconstructed radiographs) to check bony anatomy. EPID (Electronic portal imaging device) images can be used alternatively, but the sensitivity to detect setup error may be inferior to portal film. A more useful method is using the CT on rails. It is possible for CT images taken before and after SBRT to detect not only intertreatment setup error but intratreatment setup error. Recently, a couple of image-guided RT machines have been developed. With either the on-board or the in-room imaging apparatus, the position of a patient can be confirmed before every treatment day.

Target volumes and margins

Ideally, both gross tumor volume (GTV) and clinical target volume (CTV) should be geometrically defined in an unambiguous way in the reference system used. In clinical practice, however, there will always be some degree of breathing motion during imaging (even with gating there will be a residual motion) and differences in tumor position during imaging and treatment. ICRU 62 defines an internal margin (IM) and an internal target volume (ITV) for the physiologic movements and variations of the CTV during therapy. One way to get an estimate of the IM is to do the imaging during several breathing cycles (see Imaging for Planning, above). In the clinical practice of SBRT, ITV is not always defined explicitly, but PTV is usually drawn with standard margins to a CTV that has been defined by normal dose-planning imaging. The standard margins are determined from geometric verification imaging of patient cohorts and basically are valid only for the use of a particular set of conditions like patient fixation and breathing reduction, and also choice of reference system and method for setup and geometric verification. However, owing to similar geometric requirements using different methods for SBRT, a relatively narrow range of margins between CTV and PTV is currently used in clinical practice. With the immobilization equipment and methods for reduction of the target motion described in this report, the longitudinal margin is generally 10 mm. In the transverse plane, margins are usually 5 mm and up to 10 mm. Table 5 shows the margins used at different centers (16,18,19, 32,36,44,48–50,65,66).

Training requirements

The process of SBRT differs greatly from general RT in method and, more importantly, regarding patient selection, dose prescription/fractionation, target definition, and as a consequence toxicity patterns. Thus, training in SBRT is of major importance, and the following recommendations have been made: General methods for SBRT should be studied by RO (Radiation oncologist), medical physicist (MP), and radiation therapy technologist (RTT); patient selection criteria by RO; patient immobilization and accounting for internal organ motions by MP and RTT; imaging acquisition technique by MP and RTT; target definitions by RO; dose planning by MP; dose prescription by RO and MP; geometric verification by MP and RTT; treatment by RTT; toxicity patterns by RO; and follow-up by RO.

Personal experience is important not only in patient selection but also in proper use of the equipment, target definition, three-dimensional treatment planning, and follow-up of patients. Some vendors offer practical teaching courses with experienced faculty after the purchase of SBRT equipment.

QUALITY ASSURANCE REQUIREMENTS

General recommendations on quality assurance (QA) in RT also apply to SBRT. QA recommendations focused on SBRT have also been published (61), as have practice guidelines for the performance of SBRT (62). However, some aspects of QA that are of particular importance for SBRT are given below.

Table 5. Margins used for planning target volume definition for stereotactic body radiotherapy of different targets

Study	Organ	Margin transverse (mm)	Margin long (mm)	Comment	Method for breathing reduction
Timmerman *et al.*, (19)	Lung	5	10		Different methods
Bauman *et al.*, (66)	Lung	5, 10	10		Abd. comp
Zimmermann *et al.*, (65)	Lung	Individual	Individual		Abd. comp
Joyner *et al.*, (36)	Lung	5	10		
Okunieff (67)	Lung	7	10		Resp. gating
Paludan (68)	Lung	Minimum 5	10		Abd. comp
Hoyer *et al.*, (50)	Liver	Minimum 5*	10	*Later ind. margin	Abd. comp
Mendez-Romero *et al.*, (44)	Liver	5	10		Adom. comp
Wulf *et al.*, (16)	Liver	5	5, 10		Abd. comp
Kavanagh *et al.*, (48)	Liver	Minimum 5	10		Abd. comp or breath hold
Dawson *et al.*, (49)	Liver	Minimum 5*	min 5*	*Ind. margin	ABC
Svedman (69)	Liver, lung	5, 10	10		Abd. comp
Wurm (70)	Liver, lung	5	5		Adaptive gating
Hodge (71)	Lung	6	6*	*Margin to ITV	Abd. comp
Guckenberger (58)	Lung	5*	5*	*Margin to ITV	Abd. comp
Nagata *et al.*, (18)	Lung	5*	8–10*	*Margin to ITV	Abd. comp
Onishi *et al.*, (32)	Lung	0–5*	0–5*	*Margin to ITV	Different methods

Abbreviations: Resp. = respiratory; Abd. = abdominal compression. ABC = Automatic breezing control.

Treatment planning QA

Important aspects of treatment planning are adequate definitions of GTV, CTV, PTV, and OAR; conformity to dose requirements for target volumes; dose restrictions for OAR; practical aspects on a deliverable dose plan; isocenter coordinates; and accuracy in dose calculation.

The selection of adequate target volumes and an appropriate dose prescription are key factors in SBRT. Margins between GTV and CTV should be based on image information and clinical experience. The margin to PTV depends on the particular method used for SBRT, including the method for reducing internal target motion.

Evaluation of the conformity of the planned dose distribution to that intended is very important and generally requires a careful look-through of isodoses in the irradiated volume and also evaluation of dose–volume histogram data for the different volumes.

The practical aspects of the dose plan, in terms of the time for dose delivery and the possibility to reach the different beam directions, should be considered in the evaluation of the plan.

The accuracy of the dose calculation depends on the particular dose calculation algorithm used in the treatment planning system (59) and on the quantity and quality of the input data used for modeling the particular beam (radiation quality). It is important that the modeling of beam data accurately describes the beam profiles, especially with regard to geometry in the penumbra region.

Setup and geometric verification QA

The QA aspects of the geometric dose delivery are of great importance in SBRT. This can be divided into aspects of setup and geometric verification. Of importance for setup at the accelerator is that procedures for patient positioning on the treatment unit couch are the same as on the CT. Procedures to assure that the correct isocenter coordinates are used should be implemented. Preferably, this can be done with double-checking. Lasers, video cameras, imaging devices, or other equipment used for setup must be accurately aligned to the coordinate system of the accelerator (usually the mechanical isocenter (Figure). This should be checked with a phantom. The mechanical isocenter should also be checked periodically and preferably be within 0.5 mm in radius.

An important characteristic of SBRT is that direct geometric verification of the target image is used instead of imaging of surrogates for the target position, as in conventional RT. Today, several different geometric imaging methods are used in SBRT. These are CT on a device separate from the treatment unit, CT (with slit-beam or cone-beam) on a device built into the treatment unit, and projection imaging of gold markers in the tumor or of a bony tumor. For all these methods, procedures must be implemented to ensure that a proper image registration method is used to align the reference system in the geometric verification images with the same reference system in the reference image set. This procedure should be based on imaging of a phantom.

ECONOMIC CONSIDERATIONS

From the economic perspective, SBRT is more cost beneficial than surgery. In 2004, SBRT for lung tumor and liver tumors was approved by the government for insurance coverage in Japan. The charge for SBRT is only 630,000 Yen. By contrast, the surgical fee for lobectomy is approximately 900,000 Yen. The surgical fee for video-assisted thoracoscopic surgery requires both surgical and instrumental fees of 960,000 to 980,000 Yen. Other costs

including hospital charges and drug fees are higher for surgical and video-assisted thoracic surgery cases than for SBRT cases. Although similar cost comparisons for treatment in the United States and Europe have not been reported, to our knowledge, it follows that similar differences would be seen as in Japan.

SUMMARIES AND RECOMMENDATIONS

Accumulated evidence coming from the developed world strongly favors SBRT for various lung and other body tumors as an effective treatment option with acceptable toxicity (63, 64). It is expected that ongoing clinical trials will further refine its approach in selected populations of patients who are not surgical candidates for various reasons. In particular, it seems that the absolute indication of SBRT is the inoperable patient with peripheral histologically confirmed T1–3N0M0 (<5 cm) lung cancer.

However, in practice, other indicators for SBRT are encountered. Examples are an elderly patient with peripheral histologically confirmed T1–3 N0M0 (<5 cm) lung cancer who declines surgery; a patient with peripheral histologically unconfirmed (<5 cm) but radiologically diagnosed lung tumor who also declines surgery; or a patient with oligometastatic lung cancer. Other patients with primary liver cancer, oligometastatic liver cancer, pancreatic cancer, and kidney cancer could be candidates for SBRT when clinically applicable. Finally, when the patient who does not want surgery has been considered operable, SBRT can be an alternative choice.

These recommendations apply for both developed and developing countries. Moreover, several advantages inherent in the latter, such as preferred short treatment courses, fewer hospitalizations, and transport to and from hospitals, all make the cost effectiveness of this method favorable. However, certain disadvantages also exist, including high capital costs, lack of supporting (pretreatment and treatment) services, and, regardless of region, fewer patients than are usually seen in the developed world. Despite the latter, SBRT has recently been introduced in several developing countries with adequate logistics and infrastructure, which constitutes an important step toward the improvement of RT results that has been awaited for many years.

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