Increased Uptake of the Apoptosis-imaging Agent $^{99m}$Tc Recombinant Human Annexin V in Human Tumors after One Course of Chemotherapy as a Predictor of Tumor Response and Patient Prognosis

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Abstract

Purpose: Many anticancer therapies exert their therapeutic effect by inducing apoptosis in target tumors. We evaluated in a Phase I study the safety and the feasibility of $^{99m}$Tc-Annexin V for imaging chemotherapy-induced apoptosis in human cancers immediately after the first course of chemotherapy.

Experimental Design: Fifteen patients presenting with lung cancer ($n = 10$), lymphoma ($n = 3$), or breast cancer ($n = 2$) underwent $^{99m}$Tc-Annexin V scintigraphy before and within 3 days after their first course of chemotherapy. Tumor response was evaluated by computed tomography and $^{18}$F-fluoro-2-deoxy-D-glucose positron emission tomography scans, 3 months in average after completing the treatment. Median follow-up was 117 days.

Results: In all cases, no tracer uptake was observed before treatment. However, 24–48 h after the first course of chemotherapy, 7 patients who showed $^{99m}$Tc-Annexin V uptake at tumor sites, suggesting apoptosis, had a complete (n = 4) or a partial response (n = 3). Conversely, 6 of the 8 patients who showed no significant posttreatment tumor uptake had a progressive disease. Despite the lack of tracer uptake after treatment, the 2 patients with breast cancer had a partial response. Overall survival and progression-free survival were significantly related to tracer uptake in treated lung cancers and lymphomas ($P < 0.05$). No serious adverse events were observed.

Conclusions: Our preliminary results demonstrated the feasibility and the safety of $^{99m}$Tc-Annexin V for imaging apoptosis in human tumors after the first course of chemotherapy. Initial data suggest that early $^{99m}$Tc-Annexin V tumor uptake may be a predictor of response to treatment in patients with late stage lung cancer and lymphoma.

Introduction

The molecular basis of cancer is now widely believed to involve mutations that lead to deregulated cellular proliferation and suppression mechanisms controlling programmed cell death (1, 2). For instance, mutations of the powerful apoptosis-inducing myc protein or the loss of p53 protein function have been shown to play a crucial role in carcinogenesis (3). Tumor sensitivity to any given therapeutic regimen commonly is mediated by the initiation of programmed cell death via available active apoptotic pathways. Many therapeutically effective anticancer drugs act to interfere with DNA synthesis and cell division, thereby inducing apoptosis in susceptible target tumors (4, 5). Thus, it may be possible to determine the effectiveness of a proposed anticancer regimen on a patient-by-patient basis by assessing the degree of apoptosis in target tumors soon after the initial treatment.

Recombinant human Annexin V (rh-Annexin V) has been shown to bind with high avidity to PS, a membrane-associated intracellular phospholipid invariably expressed on the external cell membrane surface early in the apoptotic cascade (6). Fluorescein-labeled rh-Annexin V has been widely used as a histopathological marker of apoptosis. More recently, radiolabeled rh-Annexin V has been shown to provide noninvasive imaging of programmed cell death in animal models associated with Fas administration, organ transplant rejection, neonatal hypoxic brain injury, terminal differentiation of WBCs associated with inflammation, and cytotoxic treatment of murine lymphoma (7–14). Similarly, radiolabeled rh-Annexin V was successfully used for localizing apoptosis in human diseases such as myocardial infarction and cardiac allograft rejection (15, 16).

To assess the potential of such an imaging agent to demonstrate treatment-induced apoptosis as an early predictor to

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The abbreviations used are: rh-Annexin V, recombinant human Annexin V; PS, phosphatidylserine; CT, computed tomography; SPECT, single-photon emission computed tomography; TBR, tumor-to-background ratio; ROI, regions of interest; TR, tumor ratio; $^{18}$FDG, $^{18}$F-fluoro-2-deoxy-D-glucose; PET, positron emission tomography; NHL, non-Hodgkin’s lymphoma; HL, Hodgkin’s lymphoma; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; AI, apoptotic index; CR, complete remission; PR, partial remission; PD, progressive disease.

$^3$The abbreviations used are: rh-Annexin V, recombinant human Annexin V; PS, phosphatidylserine; CT, computed tomography; SPECT, single-photon emission computed tomography; TBR, tumor-to-background ratio; ROI, regions of interest; TR, tumor ratio; $^{18}$FDG, $^{18}$F-fluoro-2-deoxy-D-glucose; PET, positron emission tomography; NHL, non-Hodgkin’s lymphoma; HL, Hodgkin’s lymphoma; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; AI, apoptotic index; CR, complete remission; PR, partial remission; PD, progressive disease.
Patients and Methods

Patients. Fifteen patients (11 male and 4 female; mean age = 60 years) scheduled for chemotherapy of histologically confirmed NSCLC (n = 7), SCLC (n = 3), NHL (n = 2), HL (n = 1), or disseminated breast cancer (n = 2) were enrolled in this study. All patients were at least 18 years of age and clinically stable with a baseline Karnofsky Performance Status score of at least 70 and a minimum estimated life expectancy of 16 weeks. Patients were considered eligible if they had one or more extra-abdominal bidimensionally measurable lesions on an imaging study (X-ray, CT, ultrasonography, or magnetic resonance imaging) at least 1 cm in the longest diameter. All patients signed an informed consent form at moment of recruitment. Patient characteristics are summarized in Table 1.

Biochemical Basis for the Imaging of Apoptosis. The externalization of PS from the inner leaflet to the outer leaflet of the cell membrane is an universal feature occurring within 90–120 min of apoptotic signaling “in vitro,” before membrane bleb formation and DNA degradation (6, 17). Annexin V, a human protein with a molecular weight 36,000 (18, 19), which is physiologically found in the cytoplasm of a wide variety of cell types, including placental, endothelial and smooth muscle cells, is known to have a high affinity for cells with exposed PS in vitro and in vivo (20, 21).

Preparation of 99mTc rh-Annexin V. rh-Annexin V produced in Escherichia coli was labeled using a kit (Apomate; Theseus Imaging Corporation, Boston, MA) based on the performed 99mTc phentioate ligand method described by Kasina and Fritzberg (22). A radiochemical purity of $\geq 85\%$ determined by instant thin layer chromatography was required before injecting the radiolabeled tracer. 99mTc rh-Annexin V Imaging Procedure. All patients enrolled in the study were evaluated with physical examination and laboratory studies (chemistries, hematology, coagulation, routine analysis, and vital signs) before injection of the imaging agent as well as after administration of 99mTc rh-Annexin V. The protocol design is summarized in Fig. 1.

Fifteen to 30 mCi of 99mTc rh-Annexin V were administered i.v. slowly over 3–5 min. According to the study protocol, all patients underwent 99mTc rh-Annexin V scintigraphy, including planar anterior and posterior thoracic views of all measurable tumor masses at 3–6 and 24 h after tracer injection (before and after the first course of chemotherapy). Eleven patients had also dynamic sequences and whole body images immediately and 3–6 h, respectively, after 99mTc rh-Annexin V administration. Two patients had an optional SPECT acquisition 4 h after tracer injection.

Annexin V imaging was performed using a large field of view camera fitted with a low energy, parallel hole, high resolution collimator. Dynamic images were collected for 10 min after injection (20 s/frame); planar studies were acquired in a $256 \times 256$ matrix; anterior and posterior whole body images were obtained at a scan speed of 10 cm/min; SPECT data acquisition was obtained using a $128 \times 128$ matrix with a complete rotation of 360°. A minimum number of counts/images and 250,000 counts/image were collected for the planar image performed at the 3–6-h and 24-h, respectively. SPECT images were reconstructed using an iterative method based on an Ordered Subset–Expectation Maximization principle.

Image Interpretation. Qualitative assessment of tumor uptake was performed using visual reading of the Apomate images by two nuclear physicians blinded to tumor response to chemotherapy. Except for physiological distribution of tracer, any Annexin V–Tc$^{99m}$ uptake detected immediately after chemotherapy at tumor sites and not seen on the pretreatment images was considered as a positive result. Otherwise, in the absence of posttreatment tumor uptake of the apoptosis agent, the case was then interpreted as a negative result.

Semiquantitative evaluation of tumor uptake was performed in terms of TBR as well as by calculating the relative tumor uptake posttreatment compared with baseline (TR). For this purpose, several circular ROIs with similar pixel size were automatically drawn using a computerized processing (Sophy NXT, Sopha Medical). The ROIs were initially defined on the pretreatment images when an apoptotic signal was localized at the tumor sites. In a second step, these ROIs were translated on the same anatomical sites on the pretreatment images. Practically, in cases of increased Annexin V uptake after chemotherapy, the number of counts obtained for tumor-bearing Annexin V was successively noted for each ROI (i.e., ROI$_1$ (tumor signal posttreatment) = 16,000 counts and ROI$_2$ (similar tumor site pretreatment) = 8,000 counts). Thereby, allowing us to calculate the TR (i.e., ROI$_1$/ROI$_2$ = 2). Additional ROIs were also

Table 1 Patient characteristics

<table>
<thead>
<tr>
<th>Cases</th>
<th>Gender</th>
<th>Age (yr)</th>
<th>Tumor histology (stage)</th>
<th>Chemotherapy drugs</th>
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<tbody>
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<td>MIP–VP16</td>
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<td>NSCLC (IV)</td>
<td>MIP</td>
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<td>NHL (IV)</td>
<td>CHOP</td>
</tr>
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<td>MIP</td>
</tr>
<tr>
<td>6</td>
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<td>69</td>
<td>BC (III)</td>
<td>T</td>
</tr>
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<td>MIP</td>
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<td>61</td>
<td>BC (III)</td>
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<td>ABVD</td>
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<td>56</td>
<td>SCLC (III)</td>
<td>C-VP16</td>
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<tr>
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<td>Male</td>
<td>53</td>
<td>SCLC (III)</td>
<td>P-VP16</td>
</tr>
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</table>

a MIP, mitomycin-ifosfamide-cis-platinum; VIP, vepeside; CHOP, cyclophosphamide-doxorubicine-vincristine-prednisone; T, taxane; MCE, melphalan-cycloal-endoxan; ABVD, adriamycin-bleomycin-vincristine-doxorubicin; C, carboplatin; BC, breast cancer.
Define imaging of chemotherapy-induced apoptosis in patients before chemotherapy and in all patients (i.e., TBR posttreatment)/H11005. Mean values were compared using the Student’s t test when the distribution was normal and by Wilcoxon signed rank test otherwise.

The relationship of survival time to the 99mTc-Annexin V uptake (positive or negative) was estimated using the Kaplan-Meier method. The log rank test was used to compare the equality of survival curves. The degree of response to chemotherapy (CR, PR, and PD) was also appreciated in comparison to nuclear image data grading (Grade 0 to Grade 4) using Fisher’s exact test for contingency tables and for assessing the dependence between categorical variables. In addition, a linear effect between the nuclear image grading and the response to tumor therapy was analyzed using Mantel-Haenszel’s χ² test. All statistical results were considered to be significant at the 5% critical level (P < 0.05). All calculations were performed using SAS (version 6.12 for Windows) and S-PLUS 2000.

Results

Thirteen patients received two doses of 99mTc-Annexin V immediately before chemotherapy and within 3 days after completing the first course of treatment. Two patients received only one injection of Apomate after chemotherapy because of the urgency of treatment.

On prechemotherapy images, no 99mTc-Annexin V uptake was noted in tumors. Immediately before treatment, the patients only showed a similar nonpathologic biodistribution of radiotracer with uptake in salivary glands, liver, spleen, bone marrow, colon, kidneys, and bladder. On images obtained within 72 h after completing the first course of antitumor therapy, 7 patients (1 NHL, 1 HL, 2 SCLC, and 3 NSCLC) demonstrated a 99mTc-Annexin V uptake at the sites of primary or metastatic tumors. These sites were predominantly detected in regions of metastatic lymph nodes (cervical, mediastinal, and hilary nodes). Additionally, 3 patients also had a lung localization (1 NSCLC). Overall, 5 patients (1 NHL, 1 HL, 1 SCLC, and 2 NSCLC) had a significant uptake 24 h after the radiopharmaceutical injection (P = 0.02), corresponding to the 48th h after completion of the first course.
of chemotherapy, whereas 2 patients (1 NSCLC and 1 SCLC) presented with a more intense $^{99m}$Tc-Annexin V uptake 4 h after injection. The quantitative evaluation of tumor uptake in terms of TBR and TR confirmed the qualitative evaluation of the images in 6 of 7 patients having obvious tracer uptake at their tumor sites after chemotherapy. In 1 case of HL presenting with a large cervical mass, the visual interpretation showed diffuse faint uptake, whereas the TBR and TR ratios significantly differed before and after chemotherapy.

Among the 7 patients presenting with an increased Annexin V-$^{99m}$Tc uptake early after chemotherapy, 4 of them had CR (1 NHL, 1 HL, 1 NSCLC, and 1 SCLC) and the other 3 patients (2 NSCLC and 1 SCLC) had PR. On the basis of the CT and PET evaluations, all tumor sites with grade 3 and grade 4 $^{99m}$Tc rh-Annexin V uptake completely disappeared after chemotherapy (Fig. 2), whereas the subjects with grade 1 and grade 2 uptake had partial response to treatment. On the other hand, 6 of 8 patients without Annexin V tumor uptake (grade 0) after
Fig. 3 A case of NSCLC (stage IV) treated by mitomycin-cis-platinum protocol with a negative 99mTc-Annexin V study. A, thoracic CT and 18FDG PET pretreatment (1 and 2) compared with posttreatment evaluation (3 and 4) showing an obvious progression of tumor. B, first Annexin V imaging performed immediately before chemotherapy with dynamic sequences (1, top left) and static anterior and posterior views at 15 min (2, top right), 4 h (3, bottom left), and 24 h (4, bottom right) after i.v. injection of the apoptosis agent. C, second Annexin V imaging performed immediately after chemotherapy with the same protocol showing no tumor uptake but the physiological distribution of tracer (liver, spleen, and colon).
chemotherapy (4 NSCLC, 2 BC, 1 SCLC, and 1 NHL) had PD (Fig. 3). Of them, 4 patients (4 of 6) died after a median follow-up of 3 months (64–138 days). Despite the lack of significant tracer uptake after chemotherapy, two cases of breast cancer had, however, complete and partial response to Taxol, respectively. Statistically, the tumor uptake of $^{99m}$Tc-Annexin V was significantly correlated with the patient outcomes in terms of tumor response to chemotherapy and survival (Figs. 4–6).

The results of the Annexin V imaging are detailed in Table 2.

And last but not least, no serious adverse events associated with $^{99m}$Tc-Annexin V administration were observed, with a median follow-up of 4 months. In particular, no physical, biological, and hematological changes, including the coagulation tests related to the $^{99m}$Tc-Annexin V administration, were noted. However, 1 patient (case 6 in Table 2) presented a mild reaction 15 min after the second injection of $^{99m}$Tc-Annexin V (after chemotherapy) corresponding to a facial rash, which regressed spontaneously 1 h later without any treatment. This patient was still in CR at his last follow-up (76 days).

Discussion

Most anticancer drug agents as diverse as topoisomerase inhibitors, alkylating agents, antimetabolites, and hormone antagonists generate apoptosis in sensitive cells (23–25). The genetic measurement of individual components of the apoptotic pathway does not necessarily reflect the functional ability of a tumor cell to commit to apoptosis in response to chemotherapy triggering. Mutations of $p53$, for instance, block the induction of apoptosis by various chemotherapeutic drugs in many cell types, whereas newer anticancer agents such as topoisomerase poisons, particularly topoisomerase I inhibitors, are able to induce apoptosis in many cells that lack functional $p53$ (26, 27). In this study, we evaluated the technical feasibility and the clinical interest of $^{99m}$Tc rh-Annexin V for noninvasively assessing the apoptotic functional capacity of treated tumors on a patient-by-patient basis.

Preclinical studies have shown that Annexin V binds tightly to PS. PS is a phospholipid normally expressed on the inner leaflet of the bilamellar cell membrane and is invariably translocated to the outer surface as an early event in apoptosis. i.v. administered $^{99m}$Tc rh-Annexin V has been shown in preclinical and clinical studies to bind to externalized PS on apoptotic and necrotic cells with high avidity. Thus, $^{99m}$Tc rh-Annexin V uptake suggests cellular apoptosis or necrosis. Blankenberg and Strauss (7–14) showed the potential of $^{99m}$Tc rh-Annexin V for in vivo imaging of Fas-mediated fulminant hepatic apoptosis, chemotherapy-induced apoptosis in normal bone marrow and treated murine lymphoma, as well as in association with cardiac and lung allograft rejection and in hypoxic-ischemic cerebral reperfusion. Recently, in 7 patients

![Fig. 5](https://example.com/fig5.png)

**Fig. 5** Overall survival time of patients correlated with the Apomate results using the Kaplan-Meier method. $P < 0.01$ in log rank test.

![Fig. 4](https://example.com/fig4.png)

**Fig. 4** Nuclear image grading correlated with the tumor response to chemotherapy. A statistical significance was observed in both Fisher’s exact test ($P = 0.043$) and Mantel-Haenszel’s $\chi^2$ test ($P = 0.007$).

![Fig. 6](https://example.com/fig6.png)

**Fig. 6** Progression-free survival time of patients correlated with the Apomate results using the Kaplan-Meier method. $P < 0.01$ in log rank test.
presenting with documented acute myocardial infarction, Hofstra et al. (15) reported the feasibility of the radiolabeled Annexin V for the in situ imaging of necrosis and/or apoptosis. Similarly, in a series of 18 cardiac allograft recipients, Narula et al. (16) demonstrated the capability of the apoptosis imaging agent for noninvasive detection of transplant rejection confirmed by terminal deoxynucleotidyl transferase-mediated nick end labeling and/or caspase-3 (an apoptosis-specific proteolytic enzyme) immunohistochemical staining.

In oncology patients, the ability of tumor cells to respond apoptotically to chemotherapy varies from one tissue to another. In lymphomas, for instance, various treatments have shown to be efficient via chemotherapy-induced apoptosis in target tumor cells (28–31). This is one reason why this group of patients should be a priori good candidates for Annexin V imaging to assess the early apoptotic response of individual lymphomas to treatment. Indeed, 2 of 3 lymphoma patients studied in this series showed uptake of the imaging agent, suggesting their tumors had apoptotic capacity and both demonstrated objective clinical response to treatment. The third patient with a NHL showed no tracer uptake after treatment and had PD.

On the other hand, in untreated primary lung cancer, a previous work has indicated that the incidence of apoptosis known as the AI can vary widely (32). Histological analysis of 134 cases of NSCLC showed a mean AI of 0.3% (range, 0.02–1.4%), which was not correlated with the stage of disease, the degree of nodal involvement, the degree of tumor differentiation, or the differences in ploidy. Thus, the lack of significant pretreatment 99mTc-rh-Annexin V uptake in NSCLC suggests that the planar imaging technique used in this study cannot routinely detect only 0.3% apoptosis. Nonetheless, in our series, pretreatment images were obtained as a control to compare with posttreatment uptake of the imaging agent.

Similar AI data on untreated primary tumors exist for patients with breast cancer. Studies from 105 women with invasive breast cancer have shown most of the specimens from untreated patients had <1% apoptosis (33). Although no uptake of 99mTc-rh-Annexin V was seen in the 2 breast cancer patients enrolled in this series, both patients had partial clinical responses. The reason for the lack of tracer uptake visualized on imaging is not clear but may be related to failure to optimize the time of imaging after treatment when AI would have been elevated. The limited spatial resolution of currently used gamma cameras must also be considered.

Despite initial promise, in vitro sensitivity testing of tumors has not proved practical for routine clinical applications. Immunohistochemical and/or cytological apoptotic indexes determination on needle biopsy specimens showed promising results but are invasive (34). Morphological imaging procedures such as CT or magnetic resonance imaging provide accurate topographical information about the tumor changes after treatment, but they are unable to predict the response to chemotherapy. Metabolic changes usually precede gross tumor changes. PET imaging has been proposed to assess tumor viability by using either a glucose analogue (18FDG) or radiolabeled amino acids (11C-methionine, 11C-tyrosine) (35–37). However, PET performances for early imaging of the chemotherapy response can be impaired in some clinical situations by inherent biochemical and technical limitations. For instance, the possibility of cellular stunning in the first few weeks after chemotherapy, resulting in false negative results, must be considered (38).

Our preliminary results demonstrated the ability of 99mTc rh-Annexin V to localize at tumor sites immediately after the first course of chemotherapy in lung cancer and lymphoma. Biopsies were not performed in this series, and the mechanism of localization of the imaging agent must be inferred from: (a) the preclinical and clinical data showing histological evidence of 99mTc rh-Annexin V localization at sites of apoptosis and necrosis; (b) the lack of 99mTc rh-Annexin V tumor uptake immediately before treatment; (c) the objective clinical responses seen in all patients whose tumors demonstrated posttreatment 99mTc rh-Annexin V uptake; and (d) the lack of objective clinical response in 6 of 8 patients whose tumors failed to demonstrate posttreatment 99mTc rh-Annexin V uptake. For these reasons, the observations are consistent with the hypothesis that 99mTc rh-Annexin V localizes at regions of apoptosis and necrosis immediately after anticancer treatment in patients whose tumors are able to respond apoptotically to such treat-

<table>
<thead>
<tr>
<th>Cases</th>
<th>Apomate grading</th>
<th>ΔTBR a 4 h/24 h</th>
<th>ΔTR 4 h/24 h</th>
<th>Annexin V-rh-Tc99m uptake</th>
<th>Clinical response</th>
<th>Follow-up (days)</th>
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<tr>
<td>1</td>
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<td>Died</td>
<td>64</td>
</tr>
<tr>
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<tr>
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a ΔTBR (mean values), tumor-to-background ratios posttreatment (4 versus 24 h); ΔTR (mean values), relative tumor ratios posttreatment versus pretreatment (4 and 24 h); NA, not available because the patient had a single posttreatment Annexin V imaging because of the urgency of treatment.
ment. The optimal timing for scintigraphic imaging of apoptosis in this study was about 20–24 h after the second injection (48 h after chemotherapy). By taking in account the 6-h half-time of $^{99m}$Tc, the blood clearance of the $^{99m}$Tc-rh-Annexin V, as well as the counting statistics required for an adequate quality of the images, the acquisition protocol that appears the most flexible for the patient and the most efficient for imaging apoptosis after one course of treatment includes: static spot views of the thorax (anterior and posterior views) at 3–6 and 24 h after injection. Also, SPECT acquisition should probably be performed at 3–6 h after i.v. injection to improve the accuracy of detection.

This study demonstrated an excellent positive predictive value of $^{99m}$Tc rh-Annexin V imaging in NSCLC and lymphoma that warrants additional larger multicentric studies to assess in vivo the apoptotic capacity of tumors by using the apoptosis imaging agent and, consequently, tumor response to therapy on patient-by-patient basis. Although promising, the results must be, however, interpreted cautiously. The heterogeneity of the histological nature of the tumors explored, as well as the heterogeneity of the drugs administered, could explain some of the differences of tumor behavior observed. Prospective studies based on more homogeneous groups of patients in terms of histology and drugs protocol will be useful to evaluate more objectively the value of $^{99m}$Tc-Annexin V for imaging apoptosis in human tumors.

In conclusion, the preliminary results of a Phase I study demonstrated the feasibility and the safety of $^{99m}$Tc-Annexin V for localizing apoptosis in treated human tumors, particularly in lymphomas and late stage lung cancers. The selective in situ detection of the apoptotic signal is possible, as early as 1 day after the first course of chemotherapy. The determination of apoptotic competence of human tumors via their actual apoptotic response to therapy using a noninvasive and reproducible imaging tool could have important clinical implications in cancer management. Additional clinical trials based on larger multicentric series remain, however, necessary before the introduction of the technique in oncology practice.

Acknowledgments

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References


Increased Uptake of the Apoptosis-imaging Agent $^{99m}$Tc Recombinant Human Annexin V in Human Tumors after One Course of Chemotherapy as a Predictor of Tumor Response and Patient Prognosis


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