Pathologic and Immunologic Response After Ablative Radiation in Lung Cancer

NCT03603002

6/20/2020

Date: June 20, 2020
Principal Investigator: Khinh Ranh Voong MD MPH_____
Application Number: IRB00163415

JHM IRB - eForm A – Protocol

- **Use the section headings to write the JHM IRB eForm A, inserting the appropriate material in each. If a section is not applicable, leave heading in and insert N/A.**
- **When submitting JHM IRB eForm A (new or revised), enter the date submitted to the field at the top of JHM IRB eForm A.**

****

SPARC: Studying the Pathologic and Immunologic Response after Ablative Radiation in Stage I Non-Small Cell Lung Cancer

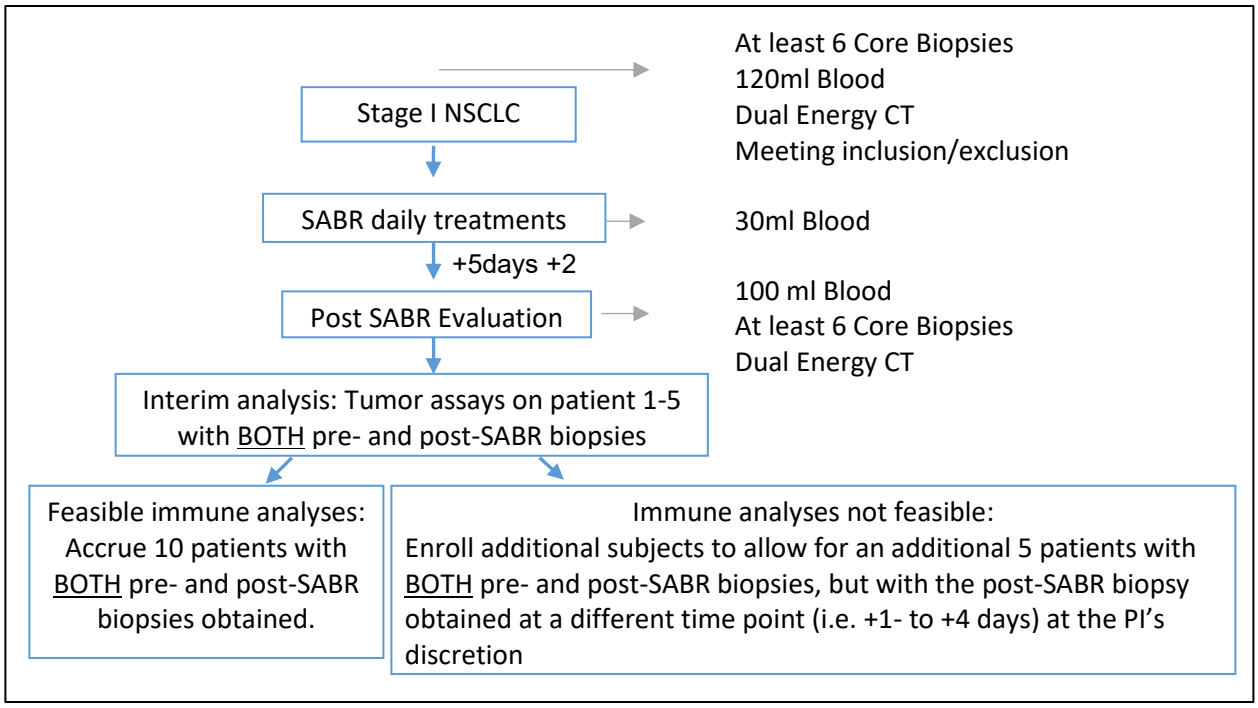
1. Abstract

a. Provide no more than a one page research abstract briefly stating the problem, the research hypothesis, and the importance of the research.

Problem: Lung cancer is the leading cause of cancer death in the United States. While stereotactic ablative radiotherapy (SABR) is delivered as standard treatment in patients with medically inoperable stage I non-small cell lung cancer (NSCLC), an alarming 30-40% of these patients still develop disease recurrence just outside of the radiation field and deadly distant metastases in their lifetime. Furthermore, since the abscopal response was reported in advanced NSCLC where a systemic cancer response was induced in areas away from the irradiated site when radiation was combined with immunotherapy, multiple clinical trials are currently investigating the role of combining these two modalities. Significantly, how SABR alone increases immunogenicity of a tumor is unknown. There is a critical need to elucidate the mechanism by which SABR alone incites the immune system to better develop future rational combinations of immunotherapy with SABR.

Hypothesis: SABR induced cell death will ultimately activate downstream cytotoxic T-cells and cause T-cell influx into the tumor to enhance immunogenic tumor cell kill. This is accomplished with SABR-induced tumor antigen—both mutation-associated neoantigen and tumor-associated antigen— release, priming of downstream cytotoxic T-cells, leading to specific T-cell clonal expansion, and resultant influx of these activated cytotoxic T-cells into the tumor and blood to enhance immune-mediated tumor cell kill.

Significance: Herein we propose a pilot study to compare pre- and post-SABR core biopsies of stage I NSCLC tumors to identify SABR-induced immune-mediated tumor recognition based on a significant and specific expansion of T-cell clones using a novel T-cell receptor (TCR) sequencing assay. This will be coupled with (1) novel genomic analysis of candidate tumor antigens that may be released from the pre-SABR tumor and (2) functional validation assays to screen post-treatment peripheral blood T-cells for reactivity to these released candidate tumor antigens. In addition, cell-based analysis will be used to identify changes in key T-cell infiltrates into the post-SABR tumor.



Study Schema

The results of this pilot study may have the potential to translate into improved systemic outcomes for patients with NSCLC through future integrated trials of immune checkpoint blockade antibodies that specifically relieve the immunosuppression on the T-cell population found to be activated by SABR. Clarifying SABR-induced immune changes in the tumor and blood will identify pathways that may be exploited to enhance systemic immunity to kill micro-metastatic disease and mitigate relapse in the next generation of clinical trials.

Additional corollary imaging studies using dual-energy (DE) computed tomography (CT), a novel imaging modality that improves the material decomposition ability of CTs, may identify new imaging markers for post-SABR treatment response by comparing DE-CT imaging characteristics with SABR fields and pathologic response.

2. Objectives (include all primary and secondary objectives)

Primary Objective:
Examine the T-cell receptor profile changes induced in the tumor after SABR.

Secondary Objectives:

1. Evaluate candidate tumor antigens (mutation associated neo-antigens, MANAs, and tumor associated neo-antigens, TAAs) released from the tumor by SABR.

2. Describe the influx of key tumor infiltrating lymphocytes in the tumor after SABR.

3. Link tumor antigen recognition by activated post-SABR peripheral T-cells with candidate pre-treatment MANAs and TAAs primed by SABR.

4. Evaluate the relationship between dual-energy (DE) CT imaging characteristics, radiation dose, and early post-SABR pathologic outcomes after treatment with SABR.

5. Evaluate the safety of post-SABR biopsies

3. Background (briefly describe pre-clinical and clinical data, current experience with procedures, drug or device, and any other relevant information to justify the research)

3.1 Disease Background and Study Rationale

3.1.1 Role of immune modulating agents in NSCLC

In the last 5 years, immunotherapy, broadly defined as a group of anti-cancer agents that aim to harness the body's own immune system to fight cancer, has transformed the management of patients with advanced NSCLC. Recently, three anti-PD-1/PD-L1 immune checkpoint antibodies — nivolumab, atezolizumab, and pembrolizumab— have been FDA-approved for the treatment of advanced NSCLC due to a survival benefit [1].

3.1.2 Role of SABR in NSCLC

SABR has quickly emerged as an important lung cancer treatment strategy as it is a promising alternative for patients with medically inoperable NSCLC or those who refuse surgical resection. SABR is thought to non-invasively and theoretically irreversibly destroy tumor. SABR allows for high doses of radiation (RT) to be given in 5 or less days, is well tolerated in the lung, and has excellent tumor control longer-term approaching 95% [23]. Despite excellent tumor control, an alarming 30-40% of patients with stage I NSCLC treated with SABR develop regional disease recurrence just outside of the radiation field and deadly distant metastases in their lifetime [2]. This is in part why lung cancer is the leading cause of cancer death in the United States [4]. Clearly, there exists a need to better define combinatorial therapies to attack occult metastases in stage I NSCLC and improve survival.

3.1.3 Clinical rationale for combining SABR and immunotherapy

A proof-of-principle trial showed an abscopal effect in patients who received granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine that stimulates APCs, in addition to RT [5]. In this trial, 41 patients with stable or progressing advanced solid tumors (including NSCLC) receiving chemotherapy also received RT and immune-modulating GM-CSF. Before enrollment, patients had 3 measurable target sites, of which 2 were sequentially irradiated. Twenty-seven percent of the patients had at least a 30% reduction in the size of the third, non-irradiated target lesion, located in a site away from the irradiated areas, with the addition of an immune-stimulating cytokine to RT. The implications of causing supra-additive systemic disease response when SABR is combined with immunotherapy is exciting in patients with early stage cancers with higher distant failure rates, such as stage I NSCLC. As such, clinicians are eagerly exploring the role of combining these two modalities.

Currently in multiple trials opened nationally, SABR is paired with immune-modulating therapies with the goal of inciting pathways that activate the body's own immune system to attack metastatic deposits of cancer [6]. SABR is used in hopes to release tumor-specific antigens and to produce a tumor-specific vaccine-like response when RT is given with immune checkpoint antibodies.

3.1.4 Study rationale

One current phase II trial open in North America that is studying pathologic tumor response to SABR, followed by resection 10 weeks following SABR [7]. Unfortunately, this trial does not evaluate immunologic endpoints as immune-related changes occur quickly. In pre-clinical mice models, RT-induced immune changes is thought to occur in the immediate days after receipt of RT [89]. Given that pathologic analysis occurs 10 weeks after receipt of SABR, this referenced trial will likely miss the immunologic changes within the tumor that may be induced by SABR.

Significantly, how SABR alone increases immunogenicity of a tumor is not fully understood, yet the investigational use of SABR given in conjunction with immune checkpoint antibodies is rapidly increasing [10]. NSCLCs are uniquely suited for studying the immune effects of SABR alone given the established role of SABR for definitive treatment of stage I NSCLC and the emerging role of SABR in the treatment of oligometastatic NSCLC[10].

There is a critical need to elucidate the mechanism by which SABR alone incites self-tumor recognition to better develop future rational combinatorial treatment with immunotherapies.

3.2 Preclinical Data

3.2.1 Effects of radiation found in the tumor microenvironment

Pre-clinical models have demonstrated that that RT strongly affects the immunologic and inflammatory milieu of cancer. In pre-clinical models, RT alone increased the number of cytotoxic effector (CD8+ CD44+) T lymphocytes present in the tumor microenvironment [11]. These activated effector T-cells are thought to be responsible for subsequent down-stream tumor cell killing. On the other hand, RT has been associated with inhibitory processes that may decrease the body's immune response to tumor. For example, RT can also enhance the presence of CD11b+ GR1+ myeloid-derived suppressor cells (MDSCs) and regulatory T-cells (CD4+ FOXP3+ Treg) in the tumor [12][13][14]. These cell types play a role in maintaining immune-suppression rather than immune activation. In mice models, RT upregulated the expression of the program death ligand 1 (PD-L1) on cancer cells [15]. PD-L1 has been shown to increase immune system suppression by binding the program death checkpoint receptor (PD-1) on T-cells [16].

3.2.2 Additional effects that radiation has on the immune microenvironment

There is growing evidence that RT treatment of a tumor create an in situ tumor vaccine by (1) inducing release of antigens during cancer-cell death in association with (2) pro-inflammatory signals that trigger the innate immune system to activate tumor-specific T-cells with the draining lymph node being the primary site of T-cell activation [17]. As such in preclinical models, RT has been linked to cytotoxic T-cell immune activation through: (1) increasing T-cell receptor interaction with tumor antigen (signal 1) by boosting the release of tumor antigens; (2) increasing appropriate positive co-stimulation (signal 2) by enhancing antigen presenting cells' (APC) presentation of tumor antigens; and (3) activating downstream effector T-cells.

RT-induced cell death releases tumor peptides that may be foreign to the host's immune system, and thus may increase signal 1. Reits and colleagues demonstrated in a mouse model that RT increased the number of peptides found in intracellular peptide pools, as well as the presentation of new tumor antigen peptides. RT increased the expression of major histocompatibility complex (MHC) class I molecules, which bind to and present bound antigens to CD8+ cytotoxic T-cells, at the cell surface days after RT in a dose-dependent fashion [18].

RT induces immunogenic cancer cell death in part by the up-regulation of calreticulin to the surface of cancer cells together with the release of high-mobility group box-1 and adenosine triphosphate. This process promotes the uptake and cross-presentation of tumor antigens by dendritic cells to T-cells in the draining lymph node [17] Pro-immunogenic signaling is accompanied by interferon β production by the dendritic cells.

RT is also shown to prime antigen presenting cells, through MHC-II, as more proliferative T-cells (CD11c+) were present in the draining lymph nodes and spleen of the mice who had ablative RT[19]. To counteract this process, a number of inhibitory immunosuppressive signals are induced including release of transforming growth factor β and colony stimulating factor 1 which result in enhanced infiltration of regulator T-cells and myeloid-derived suppressor cells. This counterbalancing determines the extent of the development of an effective antitumor immune response.

In addition, RT can improve T-cell recruitment and infiltration into the tumor by reprogramming macrophages to secrete nitric oxide leading to vascular normalization. Enhanced tumor cell secretion of chemokines, such as CXCL10 and CXCL16, recruits CD8+ T-cells to the tumor, and increased endothelial expression of vascular adhesion molecule-1 (VCAM-1) permits their extravasation [17] Once inside the tumor, RT-induced up-regulation of major histocompatibility class I (MHC-I), ICAM-1, Fas, and natural-killer group 2, member D (NKG2D) ligands on the cancer cells improves their recognition and killing by

cytotoxic T-cells [17]. NKG2D ligand up-regulation also improves NK cell-mediated killing of cancer cells that have lost MHC-I expression.

Last, RT has been linked to generation of new genetic mutations [20]. Recently, tumor mutation burden showed a positive correlation with their response to program death receptor-1 (PD-1) inhibition [21]. Thus there is a potential role of RT in increasing the tumor mutational burden and making it more immunogenic.

In summary, there is increasing pre-clinical data that RT can generate different cytokines and release tumor antigens – all which contribute to increased inflammation and potential immunogenicity of a tumor.

3.2.3 Unknown effect of SABR alone on immune response in NSCLC

Sharabi and colleagues demonstrated in vitro that a single high dose of 12 Gy stereotactic RT increased antigen-MHC complexes in irradiated cells [22]. Next, they eloquently confirmed in vivo with mice that: (1) stereotactic RT was associated with an increase in dendritic cell antigen-MHC I complexes in draining lymph nodes; and that (2) APC presentation and signal 2 were needed for RT-induced T-cell activation. By using MHC-I knockout mice, which could only provide direct antigen presentation and could not provide the co-stimulatory signal 2 with APCs, RT could not effectively activate T-cells. These results supported stereotactic RT-induced increased uptake of tumor antigens by APCs in the tumor, as well as post-RT efflux of APCs to draining lymph nodes to cross-prime T-cells. Stereotactic RT is thus being explored as an adjunctive therapy to convert a non-immunogenic, “cold” tumor to an immunogenic, “hot” tumor, through proposed mechanisms such as increasing tumor antigen release, leading to T-cell receptor engagement needed for signal 1 and increasing signal 2 within the body’s immune’s system.

Despite increasing pre-clinical data that RT strongly affects the immunologic and inflammatory milieu of cancer, the effect of RT and the corresponding influence on the tumor microenvironment is not well understood in patients. This is especially true for SABR, which delivers high ablative radiation dose and was recently developed within the last two decades. The mechanism of SABR is different than standard low-dose RT regimens studied in the past. For example, conventional fractionated RT with low daily doses of RT (1.8 – 2 Gy) has been associated with macrophage increased expression of Inducible nitric oxide synthase (iNOS), which is required for normalization of tumor vasculature [23] [24].

However, single ablative high doses of RT >10 Gy have been showed to reduce vascular flow. Reduced vascular flow impairs effector CD8+ T-cell influx into the tumor microenvironment after RT [24].

3.3 Current experience with procedures

3.3.1: Examine T-cell clonal expansion due to SABR using T-cell Receptor Sequencing.

RT enhances the diversity of the T-cell receptor (TCR) repertoire of intra-tumoral T-cells [18]. Pre- and post-SABR TCR sequencing will be performed on formalin-fixed paraffin-embedded (FFPE) tumor biopsies and peripheral blood from stage I NSCLC patients. TCR repertoire and clonality will be compared and the peripheral dynamics of intratumoral T cell clonotypes will be assessed.

In a Johns Hopkins investigator-initiated study by Drs. Patrick Forde, Kellie Smith, and Valsamo Anagnostou, TCR sequencing was used to assess T-cell clonal response after anti-PD-1 checkpoint blockade was delivered before resection. Preliminary data provided by Dr. Kellie Smith are shown in **Figure 1** below. TCR sequencing will be similarly be performed and analyzed on pre-and post-SABR tumor and blood samples.

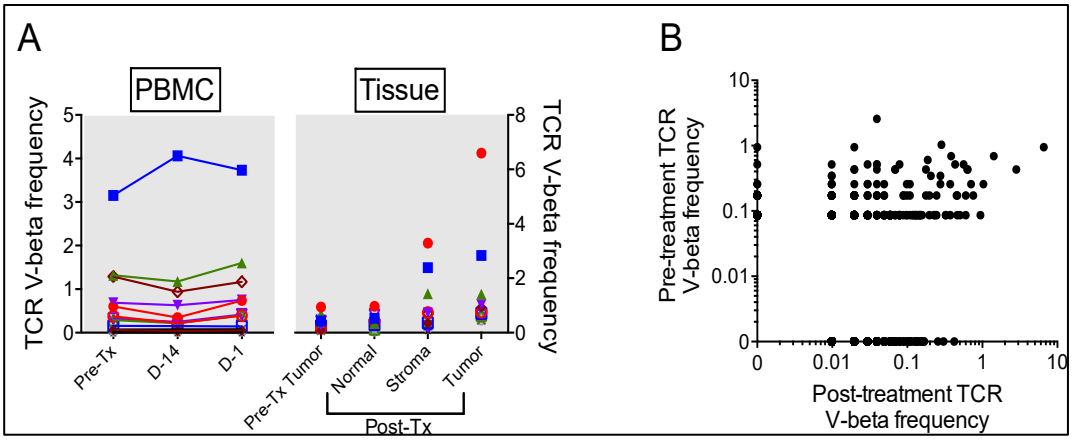


Figure 1: Alterations in TCR V-beta frequency in NSCLC patients before and after neoadjuvant anti-PD-1
Frequency in the total TCR repertoire. (A) 10 most frequent clones in post-treatment tumor. Each symbol represents a unique V-beta sequence. (B) Comparison of frequency of all clones detected before and after PD-1 in a patient with response to immunotherapy. D-14: 14 days pre-resection; D-1: 1 day pre-resection.

3.3.2: Examine candidate mutation-associated neoantigens and tumor-associated antigens released by SABR

Mutation associated neo-antigens (MANAs) are peptides that arise from the translation of unique somatic mutations in the lung cancer cell's DNA. Anti-tumor responses are integrally connected to cancer genomics, as neoantigens stemming from somatic mutations seem to shape immune responses and drive clinical benefit to immune checkpoint inhibitors. We and others have demonstrated the immunogenicity resulting from somatic mutations and shown that somatic mutational density may confer long-term benefit from immune checkpoint blockade in NSCLC [25]. When released after SABR, these MANAs may be capable of inducing an anti-tumor immune response through the above mentioned signal 1 needed for cytotoxic T-cell immune activation. Whole exome sequencing (WES) will be performed on FFPE pre-SABR core tumor biopsies to elucidate the MANAs found in the tumor that may be released with SABR. Tumor associated antigens (TAAs) are tumor antigens found across multiple lung cancers and do not develop through new somatic mutations. An increase in number of TAAs released after SABR may itself be immunogenic. RNA sequencing (RNAseq) will be performed on fresh frozen tumor pre- and post-SABR biopsies to identify potential TAAs released by SABR. These may also induce an anti-tumor immune response through cytotoxic T-cell immune activation. Dr Anagnostou has experience in performing WES and RNAseq.

3.3.3: Link antigen reactivity in the periphery with candidate antigens released by SABR.

We will evaluate reactivity of post- SABR peripheral T-cells to candidate MANAs and TAAs released by SABR. Pre-SABR tumor DNA will undergo whole exome sequencing and pre- and post-SABR tumor RNA will undergo RNA sequencing. Our neoantigen prediction pipeline will be used in combination with expression analyses to find putative immunogenic MANAs that are released or upregulated following SABR. Candidate peptide epitopes will be synthesized and used to stimulate peripheral-blood T-cells to verify functional recognition of candidate MANAs and TAAs. Dr. Smith and colleagues has experience in performing this procedure [25].

3.3.4 Examine changes in immune reactivity within the tumor pre- and post-SABR.

SABR is thought to cause ablation of all tissue within the target RT field. However immediate post-SABR tumor biopsy specimen has never been evaluated; it is therefore uncertain if immunohistochemistry (IHC) staining will reveal any identifiable remnant cell population in the immediate period after SABR. In this study, TCR sequencing of FFPE tissue is first priority. This is followed by WES of FFPE tissue in priority.

Additional FFPE core tissue will be used to evaluate the presence of leukocytes on Hematoxylin & Eosinophil (H&E) staining. If leukocytes are present, immunohistochemical staining and analysis may include but is not limited to CD4+ T-cells, CD8+ T-cells, PD-L1, and FoxP3 transcription factor.

The presence of specific key tumor infiltration lymphocytes (TILs) will first be assessed with immunohistochemical (IHC) staining of CD4 T-cells and cytotoxic CD8 T-cells. The respective T-cell density will be determined by our pathologists. We will then evaluate the presence of FoxP3, a transcription factor in CD4 T-cells, and PD-L1, a key checkpoint-related cell surface marker, in the tumor. If viable tumor cells are present, pathologists will assign an intra-tumoral and peri-tumoral immune cell infiltrate grade of (0) none, (1) rare (2) focal or (3) severe diffuse infiltration of lymphocytes. Our lung cancer pathologists will designate 3 representative fields to be evaluated by image analysis, which will allow for the data to be reported as a percentage of area (intra- and peri-tumoral) with positive T-cell infiltrate staining. Peri-tumoral versus intra-tumoral infiltrates will be scored, since these staining patterns have been shown to correlate with clinical outcomes. Pathologic response will also be scored as percentage of viable tumor, averaged from 3 areas of the FFPE-core biopsy sample. Drs. Peter Illei and Ed Gabrielson have experience in performing reviewing IHC staining of tissue.

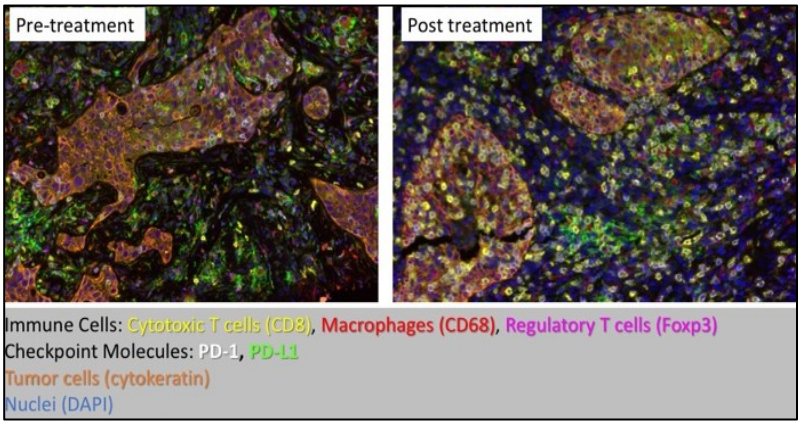


Figure 2: Immunofluorescence Pre and Post PD-1 in NSCLC patient with treatment response.

Increase in CD8+ T-cells post treatment

3.3.5: Correlate dual-energy CT imaging characteristics with radiation dose, and early post-SABR pathologic outcomes.

Dual energy computed tomography (DE-CT) is a contemporary CT acquisition technique, which permits material differentiation by three-material-decomposition mathematical algorithms. The dual energy system utilizes two sources of low and high energy KvP and the principle of different Hounsfield unit (HU) values for materials at different energies to decompose images into the components made up by different materials. This does not increase radiation dose, since the radiation is split between the two systems. For patients enrolled, CT scans of the chest with intravenous contrast will be acquired on machines with dual energy capabilities. Retrospective studies have suggested the utility of in distinguishing inflammatory tissue from tumor. NSCLC DE-CT images correlate with histologic grading of tumor [26] and can also demonstrate micro-invasiveness including vascular invasion, lymphatic permeation and pleural involvement [27]. One difficulty in utilizing SABR for treatment of stage I NSCLC is determining response to treatment. CT scans following SABR are difficult to interpret because of post-SABR lung consolidation, making accurate estimations of local failure difficult [28]. There is potential to use DE-CT as a future technique to aid in distinguishing residual tumor from post-RT inflammation. Color coded maps which are iodine overlay maps used for detection of enhancement characteristics and organ perfusion will be generated using a FDA approved post-processing software (Syngiovia, Siemens Medical Systems, Forchheim, Germany).

The utility of DE-CT after SABR in lung cancer has yet to be determined. In this secondary objective, we will evaluate DE-CT imaging characteristics using iodine uptake maps, and correlate these to gradients of high to low SABR dose from overlaid patients' RT treatment plans, as well as pathologic response. Drs. Nagina Malguria and Cheng (Tony) Lin, are thoracic radiologist with expertise in DE-CT at Hopkins.

3.3.5: Current experience with SABR

At Johns Hopkins, Drs. K. Ranh Voong and Dr. Russell Hales treat over 100 patients annually with tumors in the lung with SABR.

3.3.6: Current experience with post-SABR biopsy

Luke et. al. reports on the feasibility of post-SABR biopsy and RNA extraction as well as analysis from such samples. In this study, post-SABR biopsy was performed within 7 days after receipt of SABR on 8 patients enrolled in a phase I trial of SABR followed by pembrolizumab without reports of post-SABR biopsy-associated toxicity [29]. These 8 patients also had pre-SABR biopsy 7 days prior receipt of ablative radiation.

Feasibility of intervention after SABR may also be extrapolated from expert opinion or case series supporting the feasibility of this approach [30]. It is estimated that the incidence of in tumor recurrence after SABR is between 5 to 15%. Neri et. al. retrospectively reported on the feasibility of surgical resection as a salvage treatment for 7 patients who developed local failure from SABR with low morbidity [31]. There is one accruing clinical trial in North America that is studying the toxicity of combined approach of neoadjuvant SABR followed by surgery as a secondary endpoint [7]. This trial is scheduled to close in January 2019, with its secondary outcome of toxicity measured 7 years after the intervention.

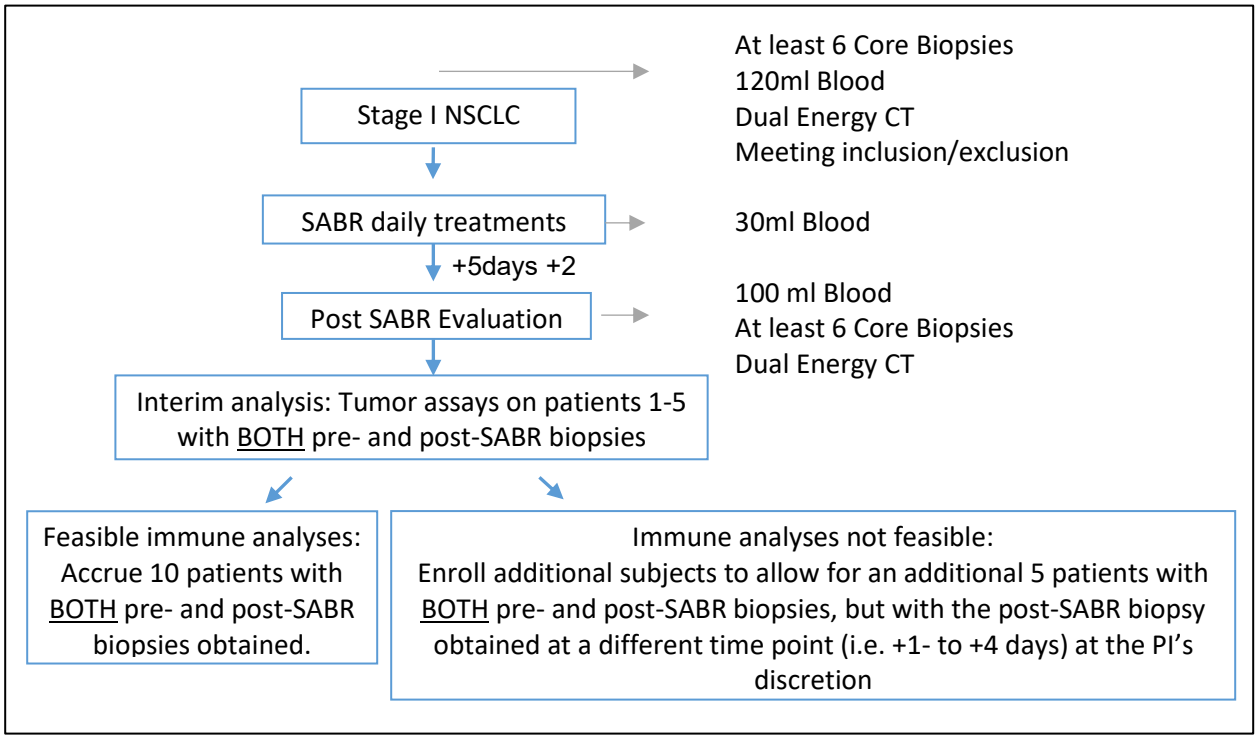
4 Study Procedures

a. Study design, including the sequence and timing of study procedures (distinguish research procedures from those that are part of routine care).

Overview of Study Design

This is a pilot study.

FIGURE 3: STUDY SCHEMA



General Guidelines

The principal investigator and all key personnel have completed NIH approved institutional and HIPAA training in the conduct of medical research studies.

Screening
Up to 40 eligible subjects will be screened. See Appendix for Screening Procedure Outline.

Informed consent is required prior to participation in this study. The study coordinator will inform potential subjects about the study opportunity and ask if they would like to participate. If the patient is interested, they will be given an opportunity to read the informed consent and authorization document specific to the study and ask questions of the study coordinator. The patient will be informed about: 1) the rationale for the study; 2) the logistics of the study; 3) the risks and benefits of the study; 4) how the data will be used. Consent will be obtained by study coordinator. The patient will be given a copy of the consent, and asked to sign another copy for our records. Patients will be registered after pretreatment evaluation is completed and eligibility criteria are met.

Recruitment

Patients will be recruited through the thoracic oncology clinics at Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins (SKCCC).

Study Process

Upon meeting study inclusion and exclusion criteria, consented patients will be registered and enrolled. Each enrolled subject will be assigned a confidential identification number (IDN).

- Baseline evaluations to confirm stage I NSCLC are to be conducted within 60 days prior to start of SABR.
    1. Medical history and physical
    2. Standard of care diagnostic core biopsies. For patients with who have already have biopsy confirmation of a stage I NSCLC completed and wish to enroll on study, they may consent to have an additional study biopsy obtained prior to SABR.
    3. Standard of care peripheral blood collection
    4. Standard of care imaging work-up including CT scan of the chest with contrast performed with dual-energy acquisition
    5. Standard of care PET-CT

    See Appendix for *Study Calendar and Procedures* and the following paragraph regarding study visits.

    In order to minimize the need for research-only in-person visits, telemedicine visits may be substituted for in person clinical trial visits or portions of clinical trial visits where determined to be appropriate and where determined by the investigator not to increase the participants risks. Prior to initiating telemedicine for study visits the study team will explain to the participant, what a telemedicine visit entails and confirm that the study participant is in agreement and able to proceed with this method. Telemedicine acknowledgement will be obtained in accordance with the Guidance for Use of Telemedicine in Research. In the event telemedicine is not deemed feasible, the study visit will proceed as an in-person visit. Telemedicine visits will be conducted using HIPAA compliant method approved by the Health System and within licensing restrictions.

- Radiation treatment planning scan will be done prior to start of SABR.

- For SABR, a total of 48 Gy will be delivered over 4 consecutive work days or 50 Gy over 5 days.

- Prior to the second SABR treatment, 30ml of study whole blood will be obtained.

- Post-SABR study peripheral blood collection and post-SABR study DE-CT will be conducted in the 0-24 hours preceding the scheduled post-SABR biopsy.

- Post-SABR study biopsy will be conducted on the 5th to 7th day after completion of SABR.
- 100cc of study blood will be obtained and stored 1 month post SABR.
- On follow-up visits, 120cc of study blood will be obtained and stored on months 3, 6, and 12 months. At the 9 month visit, 20cc of study blood will be obtained.

See Laboratory Manual, and Imaging Manual for details of the procedures.

Treatment Plan

SABR is prescribed a total dose of 48 Gy in 4 daily treatment fractions (preferred) or 50 Gy in 5 fractions (if needed per radiation oncologist's discretion). These are both standard of care SABR doses. The radiation treatment planning, prescription radiation dose, and administration will be determined by the attending radiation oncologist.

Tissue heterogeneity correction should be applied for planning. Daily on-board imaging using 3-dimensional cone beam CT (CBCT) is required before each fraction for stereotactic delivery. Additional 4-dimensional cone beam CT should be taken to verify the tumor coverage before each treatment.

General Concomitant Medication and Supportive Care Guidelines

Given a potential for interaction of high doses of anti-oxidants reducing the efficacy of radiation, the patient should abstain from the concurrent use of high dose over-the-counter vitamins, other alternative therapies, and steroids per the exclusion criteria.

Toxicity Assessments

Safety will be monitored continuously by the study investigators for the enrolled patients until study closure. All toxicities will be graded according to the Common Terminology Criteria for Adverse Events (CTCAE Version 5.0). Refer to CTCAE version 5.0 at https://ctep.cancer.gov/protocoldevelopment/electronic_applications/docs/CTCAE_v5_Quick_Reference_5x7.pdf

b. Study duration and number of study visits required of research participants.

Duration of Follow Up

Patients will be required to return for follow-up visits as per study calendar after SABR treatment. Patients will have completed the study on the day of the 1-year follow-up. They will not require additional follow-up on study, but should continue routine follow-up with treating provider.

c. Blinding, including justification for blinding or not blinding the trial, if applicable.
N/A

d. Justification of why participants will not receive routine care or will have current therapy stopped.

Duration of Therapy

In the absence of treatment delays due to adverse events, treatment may continue until one of the following criteria applies:

1. Disease progression prior to SABR,

2. Intercurrent illness that prevents further administration of treatment,

3. Unacceptable adverse event(s),

4. Patient decides to withdraw from the study, or

5. General or specific changes in the patient's condition render the patient unacceptable for further treatment in the judgment of the investigator.

e. Justification for inclusion of a placebo or non-treatment group.
N/A

f. Definition of treatment failure or participant removal criteria.

Withdrawal of Subjects from Study Treatment/On Study

- If incorrectly enrolled, subjects should discontinue study treatment and enrollment.
- Subjects may withdraw consent at any time for any reason.
- Subjects may be dropped from the trial at the discretion of the investigator should any untoward effect occur.
- A subject may be withdrawn by the investigator if enrollment into the trial is inappropriate, the trial plan is violated, or for administrative and/or other safety reasons.
- Subject is determined to have met one or more of the exclusion criteria for study participation at study entry.
- Subjects will be removed from the study if the patient cannot complete the prescribed radiation treatment or obtain post-SABR tissue required for immune analyses.
- The reason for study removal and the date the patient was removed must be documented.

Withdrawal of Consent

Subjects are free to withdraw from the study at any time without prejudice to further treatment. Subjects who withdraw consent for further participation in the study will not receive any additional study tests, but will receive standard of care follow-up to monitor adverse advents and tumor control, unless the patient has expressly withdrawn their consent to such follow-up.

A subject who withdraws consent will always be asked about the reason(s) for withdrawal and the presence of any AE. The Investigator will follow up AEs outside of the clinical study.
If a patient withdraws consent, they will be specifically asked if they are withdrawing consent to:

- all further participation in the study including any further follow-up (e.g., survival contact telephone calls)
- withdrawal of consent to the use of their study generated data
- withdrawal to the use of any samples

g. Description of what happens to participants receiving therapy when study ends or if a participant’s participation in the study ends prematurely.

If the study ends or if a participant’s participation in the study ends prematurely, patients enrolled on the trial currently receiving treatment will complete their standard of care protocol treatment, but will not receive additional study biopsy, blood work, or imaging tests. They will return for routine care and follow-up with their treating physician.

Replacement of Subjects

If a subject withdraws from the study, that subject will be replaced. In the circumstance where a study subject does not have the post-SABR biopsy obtained, that subject will be replaced. For patients who do not have the post-SABR biopsy obtained, their data will not be used in the analysis.

Protocol Enrollment Completion
Up to 40 patients will be screened. This study will complete subject enrollment and accrual when there are a total of 10 patients accrued with BOTH pre- and post-SABR biopsies obtained.

5 Inclusion/Exclusion Criteria

Inclusion Criteria

1. Written informed consent obtained from the subject prior to performing any protocol-related procedures, including screening evaluations
2. Age > 18 year
3. Histologically or cytologically confirmed non-small cell lung cancer after initial biopsies
4. Patient with accessible tumor for biopsy by transthoracic or endobronchial approach as determined by an interventional radiologist and or interventional pulmonologist.
5. Patient is to have sufficient initial core biopsy samples for tissue analyses
6. AJCC 8th edition stage I lung cancer (clinical T1a-T2a N0 M0, ≤4cm)
7. Adequate normal organ and marrow function as defined below:

Hemoglobin	≥ 9.0 g/dL
Absolute neutrophil count (ANC)	≥ 1.5 x 109/L (> 1500 per mm3)
Platelet count	≥ 50 x 109/L (>50,000 per mm3)
Creatinine	≤2 mg/dL for the dual-enhanced CT portion of the study; Creatinine is not required for inclusion into the trial

8. Patient with tumor amenable to SABR treatment as determined by a radiation oncologist
9. Subject is willing and able to comply with the protocol for the duration of the study including undergoing treatment and scheduled visits and examinations including follow up.
10. Post-menopausal status or negative urinary or serum pregnancy test for female pre-menopausal subjects. Women will be considered post-menopausal if they have been amenorrheic for 12 months without an alternative medical cause.

Exclusion Criteria

1. Primary tumors not amenable to serial core biopsies.
2. Prior thoracic radiation in the region that will be treated by SABR.
3. Patient may not be receiving any other concurrent investigational agents or chemotherapy.
4. Patient may not be receiving or received immunotherapy.
5. Patients may not be on or use steroids within 14 days before radiation, and from the duration of radiation to the time of the post-SABR biopsies and blood samples. The following are

exceptions to this criterion: Intranasal, inhaled, topical steroids, or local steroid injections (e.g., intra articular injection).

6. Female patients who are pregnant from screening to completion of SABR

See Appendix for AJCC 8 NSCLC cancer staging and Screening Procedure Outline.

6 Drugs/ Substances/ Devices

a. The rationale for choosing the drug and dose or for choosing the device to be used. -N/A
b. Justification and safety information if FDA approved drugs will be administered for non-FDA approved indications or if doses or routes of administration or participant populations are changed. -N/A
c. Justification and safety information if non-FDA approved drugs without an IND will be administered. -N/A

7 Study Statistics

a. Primary outcome variable.

Primary Objective: Examine the T-cell receptor profile changes induced in the tumor after SABR. If TCR next generation sequencing analysis of post-SABR tumor is feasible, a significant TCR profile change is defined as a multi-fold increase in the frequency of identified TCR complementarity determining region 3 (CDR3) regions present in post-SABR tumor biopsies when compared to control pre-SABR tumor biopsies.

b. Secondary outcome variables.

Secondary Objectives:

1. Evaluate candidate tumor antigens (mutation associated neo-antigens, MANAs, and tumor associated neo-antigens, TAAs) released from the tumor by SABR.

2. Describe the influx of key tumor infiltrating lymphocytes in the tumor after SABR.

3. Link tumor antigen recognition by activated post-SABR peripheral T-cells with candidate pre-treatment MANAs and TAAs primed by SABR.

4. Evaluate the relationship between dual-energy (DE) CT imaging characteristics, radiation dose, and early post-SABR pathologic outcomes after treatment with SABR.

5. Evaluate the safety of post-SABR biopsies

c. Statistical plan including sample size justification and interim data analysis.

Primary Objective:

The primary objective is to estimate the change of T cell receptor level between pre-SABR and post-SABR. A greater increase in T cell receptor level suggests a greater biological effect. With 10 analyzable patients, the change between pre-SABR and post-SABR could have an estimation precision (half of the 2-sided 90% confidence interval) of 0.58 standard deviation (with appropriate transformation when the distribution of T cell receptor level does not follow a normal distribution).

Sample Size Justification: Approximately 35 patients with medically inoperable stage I NSCLC are treated at Johns Hopkins with SABR per year and 75 patients with medically operable stage I NSCLC treated a year. We have planned an accrual rate of 1-2 patients per month and expect enrollment to be complete within 1 years.

Secondary Objectives:

For all secondary endpoints, descriptive analysis but no formal hypothesis testing will be performed given the nature of exploratory analysis. These summaries will be computed for each evaluable patient at time-points before and after SABR. Plots will be used to show the changes in immune response over time both for each individual. For each patient, comparisons in the pre and post-SABR responses will be compared using paired t-tests (or Wilcoxon signed rank tests if appropriate) for continuous variables and McNemar's test for dichotomous or categorical variables. Associations between immune responses will be explored graphically (e.g. scatterplots, boxplots) and numerically (e.g. correlations, $\chi 2$ tests).

Additional pictorial displays will be explored to evaluate the characteristics of iodine uptake after delivery of SABR in relation to SABR dose gradients.

d. Early stopping rules.

For the primary objective: To minimize unnecessary intervention, we will be monitor the post-SABR core biopsy failure rate by a Bayesian stopping rule. We will accrue patients to a different post-SABR biopsy time point, if the posterior probability of unsuccessful biopsies being larger than 70% is 70% or higher. We assume *a priori* that the post-SABR biopsy has an average of 50% successful biopsies, and there is about 22% chance that the unsuccessful biopsy rate is 70%. Based on such rules, we will assess the feasibility when 5 and 10 patients get biopsy, and determine post-SABR core biopsy is infeasible as inability to estimate T cell receptor level and change the timing of biopsies if none of 5, or less than 2 of 10 successful biopsies are observed.

For the secondary objective: To provide additional safety measures around the post-SABR core biopsy, we will monitor post-SABR core biopsy safety closely with extra caution. In particular, we will monitor the rate of unacceptable toxicity with a Bayesian stopping rule, and stop the accrual if there is sufficient evidence that the underlying rate of unacceptable toxicity is greater than 5%, e.g., the poster probability being 5% or higher is more than 80%. Unacceptable and unexpected toxicity post-SABR core biopsy is defined as life threatening hemoptysis. Life threatening hemoptysis is defined as hemoptysis requiring intubation, embolization, or admission for hypoxia. Assuming a prior that the rate of unacceptable and unexpected toxicity is very rare and has an average of 2% and there is about 11% chance that the risk will be 5% or higher. Such monitoring will stop the accrual whenever there are 2 unacceptable toxicities are observed throughout the study.

8 Risks

a. Medical risks, listing all procedures, their major and minor risks and expected frequency.

SABR Risks: In appropriately selected patients, SABR is well tolerated with low side effect profile. From prospective trial, 48 Gy in 4 fraction SABR regimen has a 13.3% adverse event rate. This was reported in a phase II study evaluating 48 Gy in 4 fractions in 45 patients who were prospectively followed for a median of 2.5 years after this regimen. Types of side effects included fatigue (11% grade 1), musculoskeletal disorders including pain (7% grade 1), injury including fracture (2% grade 2), respiratory disorders (18% grade 1, 4% grade 2) [32].

Core Biopsy Risks: The patients recruited to this study will receive core biopsies. The major expected toxicities from transthoracic or endobronchial core biopsies include self-limited asymptomatic pneumothorax (<30% with transthoracic biopsies, <2% for endobronchial biopsies), pneumothorax requiring self-limited chest tube placement or intubation (<5% for transthoracic biopsies), hemoptysis (<5%), hemorrhage (<5%), infection or other complication (<1%), and death (rare). Minor risks include pain and bleeding.

Since post-ablative radiation biopsy is not routinely performed, the increased risk of major or minor complications for biopsy after SABR is not well known and will be collected.

DE-CT Risks: There are minor risks of iodinated contrast injections (1% to 5% of headaches, nausea, and dizziness). Anaphylaxis is extremely rare (0.01%). Patients with renal insufficiency or renal dysfunction (creatinine >2 mg/dl) are excluded from the contrast-enhanced dual energy portion of study.

Peripheral Blood Draw Risks. The minor risks from additional peripheral blood draws include pain, bleeding, and infection.

b. Steps taken to minimize the risks.

To minimize risks:

Patient with stage I NSCLC tumor in an appropriate location for SBRT will be determined by the treating radiation oncologist and will be eligible for enrollment.

Patient with easily accessible tumor for biopsy by transthoracic or endobronchial approach will be determined by an interventional radiologist and/or interventional pulmonologist and will be eligible for enrollment.

Patients with renal insufficiency or renal dysfunction (creatinine ≥2mg/dl) are excluded from the DE-CT portion of the trial, but may be enrolled in the trial.

c. Plan for reporting unanticipated problems or study deviations.

The SKCCC Compliance Monitoring Program will provide external monitoring for JHUaffiliated sites in accordance with SKCCC DSMP (Version 6.0, 02/21/2019). The SMC Subcommittee will determine the level of patient safety risk and level/frequency of monitoring.

d. Legal risks such as the risks that would be associated with breach of confidentiality.

All outcomes, laboratory, radiographic, and clinical data gathered in this protocol will be handled and stored according to the Johns Hopkins Hospital data confidentiality regulations using password-protected institutional computers. All patient information will be handled using anonymous identifiers. Access to the database is only available to individuals directly involved in the study. Information gathered for this study will not be reused or disclosed to any other person or entity, or for other research. Once the research has been completed, identifiers will be retained for as long as is required by law and by institutional regulations, and at that point will be destroyed. In the rare event, should there be a breach in confidentiality, it will be handled according to institutional policies.

e. Financial risks to the participants.

Patients will be responsible their routine medical co-pays for standard of care baseline evaluation, diagnosis, treatment, and follow-up as well as associated housing and travel. Patients will not be responsible for the costs of non-standard of care procedures.

9 Benefits

a. Description of the probable benefits for the participant and for society.

The patient will receive no known additional therapeutic benefit from enrollment on this trial. However, information obtained from this study would help society better understand how SABR can affect the immune system. This knowledge would provide insight into enhancing future potential combinatorial therapies of SABR with immune modulating agents and novel biomarkers for SABR-induced immune changes. As such, this knowledge may lead to additional future treatment options combining SABR and immunotherapies that may improve the outcomes of future patients with stage I NSCLC.

10 Payment and Remuneration

a. Detail compensation for participants including possible total compensation, proposed bonus, and any proposed reductions or penalties for not completing the protocol.

There will be no compensation for participants who enroll in this protocol and no penalties for not completing the protocol.

11 Costs

a. Detail costs of study procedure(s) or drug (s) or substance(s) to participants and identify who will pay for them.

Initial evaluation including CT chest with contrast, PET/CT, blood draw, tumor core biopsies, and pathologic evaluation of mediastinal lymph nodes, in addition to SABR treatment, and follow-up are the standard evaluation and treatment protocol for patients with medically operable stage I non-small cell lung cancer (NSCLC) or for those patients who decline surgery. The costs for the work-up, treatment, and follow-up are covered by the patient's health insurance. The cost of management of toxicity of SABR treatment and post-SABR biopsies will also be covered by the patient's health insurance.

The costs of the day +5 to day +7 post-SABR core biopsies, post-SABR blood draws, and post-SABR CT scan of the chest with contrast using dual energy will be covered by the Lung Cancer Research Foundation Grant awarded to the principal investigator. The associated tests – TCR sequencing, whole exome sequencing, RNA sequencing, and peripheral blood lymphocyte antigen reactivity testing will be will be covered by the Lung Cancer Research Foundation Grant awarded to the principal investigator. The professional fees associated with radiology review of the dual-energy CT will be waived as they are performed by Drs. Tony Lin and Nagina Malguira, co-investigators, and there will be no charge to the patient.

Any additional costs, such as transportation, will be assumed by the patient.

References

1. National Cancer Institute. More immunotherapy options approved for lung cancer.

2. Timmerman RD, Hu C, Michalski J, et al. Long-term results of RTOG 0236: A phase II trial of stereotactic body radiation therapy (SBRT) in the treatment of patients with medically inoperable stage I non-small cell lung cancer. . 2014:s30.

3. Timmerman R, Paulus R, Galvin J, et al. Stereotactic body radiation therapy for inoperable early stage lung cancer. *JAMA*. 2010;303(11):1070-1076.

4. Ettinger DS, Wood DE, Aisner DL, et al. Non-small cell lung cancer, version 5.2017, NCCN clinical practice guidelines in oncology. *J Natl Compr Canc Netw*. 2017;15(4):504-535.

5. Golden EB, Chhabra A, Chachoua A, et al. Local radiotherapy and granulocyte-macrophage colony-stimulating factor to generate abscopal responses in patients with metastatic solid tumours: A proof-of-principle trial. *Lancet Oncol*. 2015;16(7):795-803.

6. clinicaltrials.gov. .

7. Palma D. A phase II trial measuring the integration of stereotactic radiotherapy plus surgery in early non-small cell lung cancer.

8. Sharabi AB, Lim M, DeWeese TL, Drake CG. Radiation and checkpoint blockade immunotherapy: Radiosensitisation and potential mechanisms of synergy. *Lancet Oncol*. 2015;16(13):e498-509.

9. Drake CG. . 2015.

10. Crittenden M, Kohrt H, Levy R, et al. Current clinical trials testing combinations of immunotherapy and radiation. *Semin Radiat Oncol*. 2015;25(1):54-64.

11. Twyman-Saint Victor C, Rech AJ, Maity A, et al. Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer. *Nature*. 2015;520(7547):373-377.

12. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*. 2009;9(3):162-174.

13. Schaue D, Xie MW, Ratikan JA, McBride WH. Regulatory T cells in radiotherapeutic responses. *Front Oncol*. 2012;2:90.

14. Sharabi AB, Nirschl CJ, Kochel CM, et al. Stereotactic radiation therapy augments antigen-specific PD-1-mediated antitumor immune responses via cross-presentation of tumor antigen. *Cancer Immunol Res*. 2015;3(4):345-355.

15. Deng L, Liang H, Burnette B, et al. Irradiation and anti-PD-L1 treatment synergistically promote antitumor immunity in mice. *J Clin Invest*. 2014;124(2):687-695.

16. Dovedi SJ, Adlard AL, Lipowska-Bhalla G, et al. Acquired resistance to fractionated radiotherapy can be overcome by concurrent PD-L1 blockade. *Cancer Res*. 2014;74(19):5458-5468.

17. Demaria S, Golden EB, Formenti SC. Role of local radiation therapy in cancer immunotherapy. *JAMA Oncol*. 2015.

18. Reits EA, Hodge JW, Herberts CA, et al. Radiation modulates the peptide repertoire, enhances MHC class I expression, and induces successful antitumor immunotherapy. *J Exp Med*. 2006;203(5):1259-1271.

19. Lee Y, Auh SL, Wang Y, et al. Therapeutic effects of ablative radiation on local tumor require CD8+ T cells: Changing strategies for cancer treatment. *Blood*. 2009;114(3):589-595.

20. Sherborne AL, Davidson PR, Yu K, Nakamura AO, Rashid M, Nakamura JL. Mutational analysis of ionizing radiation induced neoplasms. *Cell Rep*. 2015;12(11):1915-1926.

21. Yarchoan M, Hopkins A, Jaffee EM. Tumor mutational burden and response rate to PD-1 inhibition. *N Engl J Med*. 2017;377(25):2500-2501.

22. Sharabi AB, Nirschl CJ, Kochel CM, et al. Stereotactic radiation therapy augments antigen-specific PD-1-mediated antitumor immune responses via cross-presentation of tumor antigen. *Cancer Immunol Res*. 2015;3(4):345-355.

23. Tsai CS, Chen FH, Wang CC, et al. Macrophages from irradiated tumors express higher levels of iNOS, arginase-I and COX-2, and promote tumor growth. *Int J Radiat Oncol Biol Phys*. 2007;68(2):499-507.

24. Park HJ, Griffin RJ, Hui S, Levitt SH, Song CW. Radiation-induced vascular damage in tumors: Implications of vascular damage in ablative hypofractionated radiotherapy (SBRT and SRS). *Radiat Res*. 2012;177(3):311-327.

25. Anagnostou V, Smith KN, Forde PM, et al. Evolution of neoantigen landscape during immune checkpoint blockade in non-small cell lung cancer. *Cancer Discov*. 2016.

26. Lin LY, Zhang Y, Suo ST, Zhang F, Cheng JJ, Wu HW. Correlation between dual-energy spectral CT imaging parameters and pathological grades of non-small cell lung cancer. *Clin Radiol*. 2017.

27. Ito R, Iwano S, Shimamoto H, et al. A comparative analysis of dual-phase dual-energy CT and FDG-PET/CT for the prediction of histopathological invasiveness of non-small cell lung cancer. *Eur J Radiol*. 2017;95:186-191.

28. Larici AR, del Ciello A, Maggi F, et al. Lung abnormalities at multimodality imaging after radiation therapy for non-small cell lung cancer. *Radiographics*. 2011;31(3):771-789.

29. Luke JJ, Lemons JM, Karrison TG, et al. Safety and clinical activity of pembrolizumab and multisite stereotactic body radiotherapy in patients with advanced solid tumors. *J Clin Oncol*. 2018:JCO2017762229.

30. National comprehensive cancer network clinical practice guidelines in oncology. non-small cell lung cancer. . ;Version 7.2015.

31. Neri S, Takahashi Y, Terashi T, et al. Surgical treatment of local recurrence after stereotactic body radiotherapy for primary and metastatic lung cancers. *J Thorac Oncol*. 2010;5(12):2003-2007.

32. Videtic GM, Hu C, Singh AK, et al. A randomized phase 2 study comparing 2 stereotactic body radiation therapy schedules for medically inoperable patients with stage I peripheral non-small cell lung cancer: NRG oncology RTOG 0915 (NCCTG N0927). *Int J Radiat Oncol Biol Phys*. 2015;93(4):757-764.