

Molecular epidemiologic studies within the Selenium and Vitamin E Cancer Prevention Trial (SELECT)

Ashrafal Hoque¹, Demetrius Albanes⁴, Scott M. Lippman¹, Margaret R. Spitz², Phillip R. Taylor⁴, Eric A. Klein⁸, Ian M. Thompson⁹, Phyllis Goodman⁶, Janet L. Stanford⁵, John J. Crowley⁶, Charles A. Coltman⁷, and Regina M. Santella^{3*}

¹Department of Clinical Cancer Prevention and ²Department of Epidemiology, University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, USA; ³Department of Environmental Health Sciences Mailman School of Public Health of Columbia University, New York, New York 10032, USA; ⁴Division of Clinical Sciences, National Cancer Institute, Bethesda, Maryland 20892, USA; ⁵Fred Hutchinson Cancer Research Center, Seattle, Washington 98109, USA; ⁶Southwest Oncology Group Statistical Center, Seattle, Washington 98109, USA; ⁷Southwest Oncology Group, San Antonio, Texas 78245, USA; ⁸Department of Urology, Cleveland Clinic Foundation, Cleveland, Ohio 44195, USA; ⁹Division of Urology, University of Texas Health Science Center at San Antonio, Texas 78284, USA

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Abstract

Objective: To conduct timely epidemiologic investigations of molecular/genetic markers that may contribute to the development of prostate, lung, colorectal, or other cancers within the Selenium and Vitamin E Cancer Prevention Trial (SELECT), and to evaluate interactions between these markers and the study interventions.

Methods: The epidemiologic studies within SELECT will be based on 32,400 men aged 55 years or older (age 50 or older for the African-American men) enrolled into an intergroup, randomized, placebo-controlled, double-blind, phase III prevention trial of supplemental selenium and vitamin E developed and funded by the National Cancer Institute, and coordinated by the Southwest Oncology Group. During the 12-year study period approximately 1500–2000 cases of prostate cancer, 800 lung cancers, and 500 colon cancers are estimated to be diagnosed, based on data from the ongoing Prostate Cancer Prevention Trial of finasteride. A modified fasting blood sample will be processed to collect plasma for analysis of micronutrients, hormones, cytokines, and other proteins. Buffy-coat derived white blood cells collected at baseline will be used for isolation of DNA and establishment of immortalized cell lines. Red blood cells will be stored for analysis of hemoglobin adducts and other components.

Results: Specific results anticipated from these molecular studies will provide information on factors hypothesized to contribute to prostate cancer risk and that may modify the efficacy of either trial supplement, including: steroid sex hormones and several polymorphic genes that encode proteins affecting androgenic stimulation of the prostate, including the androgen receptor, steroid 5 α -reductase type II, CYP17, and β -hydroxysteroid dehydrogenase; polymorphisms of DNA repair genes and carcinogen metabolism genes, including those involved in the activation of chemical carcinogens to reactive intermediates (e.g., CYP1A1) or the detoxification of reactive intermediates (e.g., glutathione S-transferase M1); DNA and protein adducts; and insulin-like growth factors and leptin.

Conclusion: SELECT offers an excellent opportunity to conduct molecular epidemiologic investigations to assess gene–environment interactions and their role in prostate, lung, and colon carcinogenesis.

* Address correspondence to: Regina M. Santella, Mailman School of Public Health, Columbia University, 701 West 168th St (Room 505), New York NY 10032, USA. Ph.: (212) 305-1996; Fax: (212) 305-5328; E-mail: rps1@columbia.edu

Reprint requests to: Southwest Oncology Group, 14980 Omicron Drive, San Antonio, TX 78245-3217, USA

Introduction

The Selenium and Vitamin E Cancer Prevention Trial (SELECT) is a large, intergroup randomized, phase III trial developed and funded by the National Cancer

Institute, and coordinated by the Southwest Oncology Group. The primary objective of the study is to assess the effect of supplementation with selenium and vitamin E, alone and in combination, on prostate cancer incidence in a double-blind, placebo-controlled fashion. The target sample for this 12-year trial is 32,400 healthy males, age 55 years or older (age 50 or older for the African-American men). The study will encompass a 5-year accrual period, with a minimum of 7 years of intervention. An overview of the rationale and design of SELECT has been published [1]. Secondary objectives of SELECT are to assess the effect of the interventions on incidence and survival of other cancers (lung, colon, and all cancers combined) and on cardiovascular events.

One of the tertiary objectives of SELECT is to conduct timely molecular epidemiologic investigations during and after the trial, based on blood specimens collected at baseline from all participants, and from a subsample annually. The overall goal is to evaluate molecular/genetic markers that contribute to the development of prostate, lung, and colorectal cancers in particular, and to assess intervention efficacy according to the status of these markers. This paper outlines the objectives, procedures, and key *a-priori* hypotheses to be tested through the molecular epidemiologic studies within SELECT.

Molecular epidemiologic studies

Nested case-control studies will generally be utilized for the analysis of SELECT biospecimens, with incident cancer patients being matched to participants free of cancer at the time of the cases' diagnoses. Since all SELECT enrollees will have blood drawn at baseline and be on-study for up to 12 years, the nested case-control design is highly efficient and affords the advantage of providing sample-based risk estimates that are as stable as those generated from analysis of the entire cohort.

All incident cases of prostate, lung, and colon cancer within the SELECT cohort will be identified by the Statistical Center and made available for inclusion in the nested investigations. Approximately 1500–2000 prostate, 800 lung, and 500 colon cancer cases are estimated to occur, based on data from the ongoing Prostate Cancer Prevention Trial of finasteride, during the 12-year study period. For the purposes of the case-control studies, cases will be compiled at three time points during the trial (*i.e.*, at the end of 4, 8, and 12 years), and controls will be matched to cases based on age, ethnicity, and length of follow-up. Investigations involving mi-

cronutrient assays may also match for date of blood collections (*e.g.*, for season). Linkage to questionnaire and biospecimen data for statistical analysis will follow.

Risk factor data

After study subjects give informed consent and are enrolled into the trial, data will be obtained on sociodemographic characteristics including age, race, ethnicity, and other risk factors such as smoking history, diet, alcohol and vitamin supplement use, body weight, physical activity, and family history of cancer, particularly in first-degree relatives. Completed questionnaires will be forwarded to the Statistical Center for editing and data entry.

Biospecimen collection and processing

A modified fasting blood sample will be collected from all participants at the time of randomization. Blood processing will be carried out to provide: (1) plasma for analysis of micronutrients, hormones, and other proteins and analytes of interest; (2) buffy-coat derived white blood cells (WBC) for the isolation of DNA and viable lymphocytes for transformation and establishment of cell lines that will provide an unlimited source of DNA for future studies and the possibility to conduct functional, cell-culture assays (*e.g.*, DNA repair activity); and (3) red blood cells (RBC) for analysis of hemoglobin adducts and other components. Toenail samples will also be collected. Additional blood will be obtained annually from approximately 6% of the participants for compliance assessment and comparison of biomarker data across time using procedures similar to those used at baseline, and outlined below.

Approximately 20 ml of blood will be collected into three 7 ml acid-washed vacutainer tubes containing EDTA as anticoagulant, and protected from the light. The SELECT study protocol will indicate that bloods should be obtained at least 3 hours after a meal (*i.e.*, a modified fast), and the date and time of blood collection, as well as time since last meal, will be documented. To maintain the integrity and minimize necessary handling/processing of the samples obtained for this study of over 32,000 individuals, it is important that the samples be transported in an efficient manner that preserves the key biomarkers of interest (*i.e.*, micronutrients, hormones, proteins, and viable lymphocytes). This is particularly true in that they will be collected throughout the year at sites across North America. Modified ice-pack styrofoam shipping boxes will be utilized to keep blood chilled but not frozen (*e.g.*, 2 °C)

during transport, thus preserving both plasma components and lymphocyte viability. A pilot study of these procedures is in progress.

Blood samples will be shipped by overnight delivery to the NCI-Frederick Cancer Research and Development Center for processing and storage. Plasma will be divided into 0.5 ml aliquots and frozen at -70°C . Buffy-coat WBCs will be collected and separated into four aliquots: two aliquots consisting of about 75% of the cells will be frozen in vapor-phase liquid nitrogen for later DNA extraction, with the remainder having dimethyl sulfoxide (DMSO) added before aliquotting, freezing at controlled rate, and transferring to liquid nitrogen for future EBV transformation of lymphocytes and establishment of cell lines. Two large aliquots (5 ml) of RBCs will also be stored (-70°C).

Molecular/genetic markers

The first analyses of SELECT specimens will not be carried out until 4 or 5 years after the trial has begun. This, coupled with the certainty of new hypotheses evolving from our present knowledge regarding prostate carcinogenesis; the identification of new candidate genes and other exposures that may influence susceptibility; and biotechnological improvement in the efficiency of analysis, sensitivity, and reproducibility of assays, argues in favor of a flexible analytical plan for these investigations. For example, automated systems will facilitate rapid isolation of DNA from lymphocytes, and advances in microchip technology will permit rapid genotyping of multiple polymorphisms. These systems are being validated by comparison to data generated by standard polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) methods and will likely be operational by the time genotyping will be carried out on SELECT samples.

Several biomarkers of relevance to prostate carcinogenesis that will likely be evaluated *a priori* in SELECT are discussed below, many of which are also active, testable hypotheses for cancers of the lung and large bowel.

Sex hormones

There is considerable evidence that steroid hormones play a critical role in controlling cell proliferation in several cancers, including prostate cancer. The prostate is hormonally regulated by testosterone, which is converted into its reduced and metabolically more active form, dihydrotestosterone (DHT), through the activity of steroid 5α -reductase type II (*i.e.*, SRD5A2). Both hormones bind to the androgen receptor (DHT with

greater affinity), and form a complex that translocates to the nucleus, binds DNA, and transactivates genes having androgen-response elements.

Plasma androgens (*e.g.*, testosterone and androstenedione) and other sex hormones will be measured and related to prostate cancer risk and, at the end of the study, to the trial interventions. Direct assessments of human exposure to endogenous steroids is made difficult, however, by assay variability, individual circadian and sporadic variation in circulating hormone concentrations, and the often imprecise relationship between circulating, target tissue, and intracellular hormone concentrations [2]. It is therefore imperative to assess polymorphic genes related to androgen metabolism with the understanding that knowledge of their impact on an individual's hormonal milieu will enhance our interpretation of relationships with circulating hormone levels.

In a prostate cancer model developed by Ross and colleagues, four genes have been targeted for investigation [2]. These polymorphic genes encode proteins that affect androgen stimulation in the prostate and show allelic variation among racial-ethnic groups. They are the androgen receptor (AR), SRD5A2, cytochrome P450c 17 α (CYP17), and β -hydroxysteroid dehydrogenase (HSD3 β 2). As mentioned, the AR encodes the receptor for androgen binding and transport, DNA binding, and transactivation of genes with androgen response. SRD5A2 is responsible for the conversion of testosterone to the metabolically more active dihydrotestosterone. The enzyme CYP17 regulates critical steps in testosterone biosynthesis. HSD3 β 2 encodes one of the two β -hydroxysteroid dehydrogenase enzymes involved in the metabolism of dihydrotestosterone in the prostate and the catalysis of a critical step in testosterone biosynthesis. It is likely that these genes – and others to be discovered – involved in androgen biosynthesis and androgen activation, transport, and metabolism in the prostate will be important determinants of prostate cancer risk.

One of the planned investigations within SELECT is of the CYP17 polymorphism. This gene codes for the cytochrome P450c17 α enzyme, which mediates both steroid 17 α -hydroxylase and 17,20-lyase activities, and plays a key role in human steroidogenesis [3]. The variant allele (A2) has a single base-pair substitution (T \rightarrow C, identified by the MspA1 restriction enzyme) in the 5' promoter region of exon 1, which creates an additional Sp1-type (CCACC) promoter site and may enhance transcription [4]. This led to the hypothesis that the A2 allele may be associated with elevated testosterone production. However, conflicting evidence regarding the molecular biology of this polymorphism has been reported [5, 6].

A preliminary report found that men homozygous for the wild-type A1 genotype had significantly higher serum androgens than men with a least one A2 allele [7]. In a Swedish case-control study of men referred to three hospitals with prostate cancer and population-based men without the disease, the A1/A1 genotype was associated with a significant elevation of prostate cancer risk (odds ratio, OR = 1.6; 95% confidence interval 1.0–2.5). These authors suggested that the A1/A1 genotype may confer a more active androgen synthesizing CYP17 enzyme [8]. However, another group of investigators reported inconsistent results. Lunn *et al.* [9], in a urology clinic-based case-control study, found that men with at least one A2 allele (A1/A2 or A2/A2 genotype) had an increased risk of prostate cancer (OR = 1.7, 95% CI 1.0–3.0). This enhanced risk associated with the A2 allele agrees with the hypothesis that this variant may increase testosterone production. Interestingly, the association with the A2 allele was strongest for men diagnosed at an earlier age (OR = 2.3, 95% CI 1.0–5.4). The later study, however, used urology clinic controls with conditions that may be related to androgen levels. Thus, it is difficult to interpret these results. Given the importance of the CYP17 gene in androgen production, further study of this polymorphism is warranted.

Carcinogen metabolism and DNA repair

Numerous molecular epidemiologic studies have investigated the role of polymorphisms of carcinogen metabolism genes, including those involved in the activation of chemical carcinogens to reactive intermediates (*e.g.*, CYP1A1) or the detoxification of reactive intermediates (*e.g.*, glutathione *S*-transferase M1, or GSTM1) [10–12] in human carcinogenesis. They demonstrate that several polymorphisms modify risk for breast, lung, and bladder cancers, but data concerning their impact on prostate cancer risk are limited. For example, a study by Harries *et al.* demonstrated a highly significant decrease in the frequency of the glutathione *S*-transferase P1a homozygous genotype in prostate cases compared to controls [13]. In addition, polymorphisms in DNA repair genes have recently been identified [14]. Although the phenotypic expression of these polymorphisms has not been clearly defined, work has begun in a number of laboratories to determine if they influence cancer risk. Already the first reports have been published regarding the association between polymorphism in the XPD gene and basal cell carcinoma [15] and XRCC1 and squamous cell carcinoma of the head and neck [16]. Further evaluation of genes encoding DNA repair enzymes, as well as phase 1 and phase 2

metabolism enzymes, will likely be a major focus of research within SELECT ancillary investigations of genetic susceptibility factors.

DNA and protein adducts

DNA adducts, resulting from covalent modification by chemical carcinogens, are frequently elevated as markers of exposure and susceptibility [17–19]. Leukocytes can be used for measurements of DNA adducts using ³²P postlabeling, immunohistochemistry, or other techniques which require small amounts of DNA or small numbers of intact cells. As a surrogate for DNA adduct measurement, protein adducts are also frequently measured [20].

While the prospectively collected samples in this trial could be used to study numerous environmental exposures, an example of such adducts of interest are those resulting from exposure to polycyclic aromatic hydrocarbons, or PAHs. Human exposure to PAHs, including benzo[*a*]pyrene, is associated with an increased rate of lung, skin, and bladder cancer [21], and with increased mortality from causes related to atherosclerosis [22]. Such adducts will be evaluated as environmental exposures and in relation to susceptibility genes (*i.e.*, for gene-environment interaction). The rate of PAH adduct formation is partially related to genetic polymorphisms in PAH metabolizing enzymes such as CYPs and glutathione *S*-transferases (GST's) (*e.g.*, refs. 23 and 24). Variations in PAH metabolism, and consequently cancer risk, have been associated with specific genotypes [25] and will be investigated.

Insulin-like growth factors

Insulin-like growth factor-1 (IGF-1), a polypeptide with structural homology to insulin, has been positively associated with adult long bone growth, decreased lean body mass, increased body mass index, and increased abdominal obesity [26–29]. Recent evidence also suggests a role for IGF-1 in tumorigenesis. IGF-1 has mitogenic effects *in vitro* on numerous types of cancer cells, including human prostate cancer cells [30], and high plasma IGF-1 levels *in vivo* are associated with increased risk of prostate cancer [31, 32]. Recent studies have reported racial differences in IGF-1 and binding protein-3 (IGFBP-3) in men at increased risk of prostate cancer [33, 34]. IGFBP-3 plasma levels were found to be lower in African-American men than Caucasian men [33, 34]. This finding may help explain in part the reason for increased risk of prostate cancer in African-American men. Studies have also shown that the tumor-inhib-

iting effects of calorie restriction in rodent tumor models are mediated in part by reduced plasma IGF-1 levels [35, 36]. IGF-1 has also been linked to reproductive hormones and leptin. Increased plasma IGF-1 levels, which are found in obese women, decrease sex hormone-binding globulin levels, which in turn increase the levels of unbound sex hormones [37]. In addition, obese individuals with increased insulin levels have lower plasma levels of IGF-binding proteins, allowing greater local activity of IGF-1 in the ovary, endometrium, and breast [37]. The ratio of plasma IGF-1 to IGF-BP-3 was correlated with fasting plasma leptin levels in a study of 19 centenarians [38]. In cultured rat adipocytes and ovarian granulosa cells, IGF-1 inhibits the expression of *ob*, the rodent equivalent of leptin, in a dose-dependent manner [39, 40]. Thus, measurement of IGF-1 and IGFBP-3 may provide insight into prostate cancer etiology.

Leptin

Leptin is a 16 kDa peptide hormone secreted from adipocytes involved with appetite control and energy metabolism through hypothalamic influence. The primary physiologic role of leptin appears to be the maintenance of energy homeostasis through signal feedback to the central nervous system regarding the size of fat stores [41]. In the non-obese state, rising leptin levels result in decreased appetite and increased energy metabolism through a series of neuroendocrine changes. The obese state is associated with high circulating leptin levels [42–46], suggesting that obese individuals may be leptin-resistant. The mechanism underlying this apparent leptin insensitivity is not fully understood, but may be due to a defect in leptin signal transduction, resulting in a higher “set-point” of body weight [41]. Recently, Changnon *et al.* reported a significant effect of leptin receptor on adiposity in middle-aged Caucasian men [47]. The only published report of circulating leptin levels and cancer risk, a small case-control study of prostate cancer, suggested less likelihood of positive association [48]. In as-yet-unpublished studies, significantly higher leptin levels have been observed in men with high-volume prostate tumors than in patients with low-volume tumors (Chang *et al.*, unpublished), suggesting that leptin may be involved in prostate cancer progression.

Other related investigations

Additional ancillary studies nested within SELECT will focus on nutrition-related factors and tumor markers. Baseline dietary nutrient intake and supplement use and

plasma micronutrients, particularly vitamin E and selenium, will be assessed in order to test both their direct associations with prostate cancer risk, and whether the effects of the two trial supplements on prostate cancer incidence are conditional on pre-randomization intake or biochemical status. Other timely diet-related hypotheses will also be investigated, including the role of dietary fat and fatty acids, lycopene and other carotenoids, meat, and soy, for example. As mentioned, plasma vitamin E and selenium levels will also be measured annually during the trial in a cohort subsample for the purpose of monitoring study adherence.

Tissue-based studies of tumor markers will also be conducted. Potential molecular/genetic markers of interest include proliferation markers such as Ki-67, invasion markers such as MMP2, MMP9, stromelysin, cathepsin D, and E-cadherin, cell cycle proteins such as cyclin A, cyclin D, cyclin E, p21, p16, p27, p53, and Rb, and mutations in genes associated with tumor progression.

Resource availability

Biospecimens will be available to SELECT investigators as well as to outside researchers with important, timely hypotheses. Because the collected biospecimens represent a limited resource, proposed investigations will be evaluated in terms of scientific significance and validity, as well as their potential impact. The amount and type of material requested will also be considered, to ensure the efficient use of samples. Potential investigators will be asked to submit a brief proposal abstract and 1–4-page outline describing background, hypothesis, and methods. Proposals will be circulated for review by the Molecular Epidemiology Subcommittee of SELECT and two outside *ad-hoc* members not affiliated with the Southwest Oncology Group who have relevant expertise in cancer genetics and molecular biology. It is anticipated that proposals will be reviewed once a year. Approvals from both the Subcommittee and the investigators' Institutional Review Board will be required prior to release of samples. Also, investigators planning to submit NIH grant applications must obtain approval for their study and specimen access from the SELECT Molecular Epidemiology Subcommittee before submission of a grant proposal.

Future directions

It is likely that, over the next 10 years, advances in molecular biology and genetics and newly emerging technology will dramatically impact which methodologies and markers are of interest in the SELECT cohort.

Thus, additional molecular hypotheses will be developed for testing within SELECT through nested case-control investigations. End-of-study evaluation of the effects of the trial supplements on prostate cancer incidence in the context of specific genetic backgrounds and other molecular markers will also be important.

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