## A) FINAL PROGRESS REPORT

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## List of Terms and Abbreviations:

ELISA, enzyme-linked immunosorbent assay; SELDI-TOF MS, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry; TGF$\beta$1, transforming growth factor beta-1

# P53 Biomarker and Intervention in Occupational Cancer

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Program Director/Principal Investigator: Paul W. Brandt-Rauf

## Abstract

One of NIOSH's stated needs in the area of research methods for occupational cancer has been developing early markers of adverse health effects from workplace exposures and devising ways for interrupting the pathways between workplace exposures and resultant cancers. A potential model population for studying these issues is provided by asbestos-exposed workers for which there is a critical need for ways to detect those workers most at risk for the development of asbestos-related cancers as well as interventions to successfully treat and prevent their cancers. Thus, the purpose of this research has been two-fold: (1) to develop new biomarkers which will have high predictive value for the subsequent occurrence of asbestos-related disease, with a particular emphasis on the tumor suppressor gene p53 which is known to be altered by asbestos in many cases; and (2) to develop new interventions that could treat and prevent asbestos-related cancers that have an altered p53 gene, such as many lung cancers and mesotheliomas.

For the first goal, we utilized the banked serum samples from a cohort of Finnish asbestosis cases who have been followed up for the occurrence of cancer to examine p53 auto-antibodies, since in many cases where p53 is mutated individuals develop an immune response against the structurally altered mutant protein. We were able to demonstrate that p53 autoantibody biomarkers do have significant predictive value for the development of cancer in asbestosis cases and in fact correlated with the changes in p53 found in the subsequent cancers; however, the sensitivity of this single biomarker was somewhat low. Therefore, we proceeded to use proteomic technology for additional biomarker discovery in the banked serum samples to improve the sensitivity for cancer detection. Preliminary results on a small sub-set of these samples suggested a proteomic profile in these samples of high sensitivity and specificity. One specific peak corresponded to transforming growth factor beta-1 (TGF$\beta$1). An ELISA-based assay was then used to examine TGF$\beta$1 in all samples, but statistical analysis showed that it was not specifically correlated with the development of cancer but rather with the severity and progression of the asbestosis. Subsequent proteomic analysis of all the samples from asbestos-related cancer cases and non-cancer controls yielded a different battery of protein biomarkers with high sensitivity and specificity as well as predictive value for the carcinogenic effects of asbestos exposure. Two of these peaks were identified as kinesin-family proteins, which could be plausibly related to both asbestos exposure and altered cell division leading to cancer. For the second goal, we initially constructed unique protein sequences from p53 that can cause cell death in mutant p53 pre-malignant and malignant cells (including lung cancer cells similar to those that occur in the asbestosis cohort) in cell culture when delivered as the peptide or as a mini-gene. Subsequently, we demonstrated the effectiveness of the most promising of these p53 peptides as therapy *in vivo* in animal models of nude mice xenografted with the same mutant p53 cancer cells. Such peptide therapies could interrupt the p53-dependent carcinogenic pathway between asbestos exposure and resultant cancers. Taken together, these results can be utilized in the workplace for the improvement of secondary prevention of asbestos-related cancers. For example, workers in high-risk cohorts due to their exposure can be screened with a battery of biomarkers (including p53 antibodies and kinesin proteins) to detect those individuals with the greatest likelihood of developing subsequent malignant disease. Workers who are identified by the p53 biomarker would then be candidates for a preventive intervention based on the p53 peptide therapy before clinical cancer develops. The intervention could also be used for those asbestos workers who have already developed a

cancer with altered p53. The success of the treatment or prevention could be monitored by following the biomarker status.

**Section 1**

Highlights/Significant Findings:

This research has two highly significant findings. First, it has identified several important new serum biomarkers for the development of occupational cancer caused by asbestos exposure. Second, it has identified a new therapeutic/prophylactic approach to treat/prevent many of the cancers caused by asbestos exposure.

Translation of Findings:

The findings of this research can be translated for use in the early detection, treatment and prevention of occupational cancers related to asbestos exposure. Among asbestos workers at any given level of exposure, it is known that not all will develop cancer. The newly identified biomarkers can be used to identify those workers who are at the greatest risk of asbestos-related cancers. These workers can then be targeted for more aggressive interventions to prevent the occurrence of cancer based on the exact genetic defects identified by each particular biomarker. A p53 peptide-based approach is such an intervention which has proven successful in cell culture and animal studies and is now ready to proceed to human investigations.

Outcomes/Relevance/Impact:

The results of this research have significant potential outcomes which could impact workplace risk if used, as suggested above. In particular, the results could lead to better secondary prevention of occupational cancer by identifying asbestos-exposed workers at the highest risk for subsequent development of cancer who could be targeted for more aggressive interventions directed at the specific genetic defects indicated by the biomarkers. In particular, those workers with p53-positive biomarkers indicative of elevated cancer risk could be targeted for the specific therapeutic/prophylactic intervention based on the p53 peptide that would treat or prevent the cancers, thus reducing asbestos-related cancer morbidity and mortality.

**Section 2**

Scientific Report

According to the original NIOSH National Occupational Research Agenda, new Research Methods for Occupational Cancer are needed to develop knowledge that can be used in preventing occupational cancers and to better understand their underlying pathophysiology. These methods include measuring early markers of adverse health effects as early warning systems for exposed populations and finding new ways and approaches for interrupting the etiologic and natural pathways between an occupational exposure and resultant cancers. Advances in recent years of our understanding of the molecular biology of carcinogenesis offer new opportunities for addressing both of these aspects of occupational cancer research in a coordinated fashion, i.e., developing new molecular markers of early carcinogenic effects from

workplace exposures and developing new interventions designed to attack the specific molecular defects detected by these biomarkers. An initial target of this research for the development of such biomarkers and interventions was the p53 tumor suppressor gene product in asbestos-related malignancies due to the fact that p53 is the most frequently identified site for mutations in human cancers, including those caused by asbestos exposure such as lung cancers and mesotheliomas.

It was also known that individuals with tumors that contain mutant p53 can mount an antibody response directed against the mutant protein, presumably due to the conformational alterations produced by the mutations which cause it to be identified as "foreign" by the body's immune system, and that in some cases the detection of p53 autoantibodies can precede the occurrence of clinical disease. Thus, p53 antibodies were considered to be a potential useful early biomarker of carcinogenic risk for cancer including occupational cancers from asbestos exposure. Therefore, initially the research focused on the analysis of the presence of p53 autoantibodies using an enzyme-linked immunosorbent assay (ELISA)-based approach in the multiple banked serum samples (268 total samples or 1-5 samples per worker collected between 1980-1988) from a cohort of 103 Finnish asbestos workers with compensable asbestosis who were followed up for the subsequent development of cancer through the Finnish Cancer Registry (a total of 49 cancers including 31 lung cancers and 4 mesotheliomas). The results demonstrated a highly statistically significant relationship between the presence of p53 autoantibodies in the serum samples and the subsequent development of cancer in the workers (Hazard Ratio=5.5, 95% Confidence Interval=2.8-10.9), controlling for age, gender, smoking and cumulative asbestos exposure, with a positive predictive value of 0.76 and an average lead time to diagnosis of 3.5 years (range=<1-12 years). The presence of p53 autoantibodies in the serum samples was also statistically significantly associated with p53 alterations in the resultant tumors (kappa=0.78, p=0.01). Thus, p53 autoantibodies could be a useful early biomarker of cancer risk in asbestosis cases and could be used to identify high-risk individuals who could benefit from more aggressive preventive interventions based on p53-specific pathways (see below). However, although the specificity of this biomarker was high (0.93), its sensitivity was lower (0.27). Therefore, in the subsequent phases of this research, we decided to apply proteomic approaches for additional biomarker discovery in the banked serum samples to improve the sensitivity for cancer detection.

Using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS), we initially examined the proteome differences in single serum samples from 5 of the asbestosis cases who developed cancer and 5 matched cases who remained cancer-free. These preliminary results suggested that transforming growth factor beta-1 (TGFβ-1) might be discriminatory for subsequent malignant disease. We therefore established an ELISA assay for TGFβ-1 and analyzed all of the serum samples. However, levels of TGFβ-1 were not found to correlate with the subsequent development of cancer. Nevertheless, the levels of TGFβ-1 were found to be statistically significantly related to the radiographically determined level of asbestosis disease severity (p=0.01, with TGFβ-1 levels increasing approximately 2.4-fold from ILO radiographic category 0 to category 3) and to be marginally related to the occurrence of asbestosis disease progression over the course of the study (p=0.07), after controlling for other contributory factors including cumulative asbestos exposure. These results suggested that serum TGFβ-1 might be a useful biomarker of asbestos-induced non-malignant fibrotic disease. In order to continue to look for potential cancer-related protein biomarkers, we then applied a refined SELDI-TOF approach with classification and regression tree (CART)

statistical analysis to the proteomic examination of serum samples from all of the asbestos-related cancer cases (lung cancers and mesotheliomas) and matched non-cancer cases. These results indicated three differential protein peaks (5707.01, 6598.10 and 20,780.70 Da) that could predict the subsequent development of cancer with 87% sensitivity and 70% specificity. Using standard protein enrichment and isolation techniques, the first two of these protein peaks were identified as KIF18A and KIF5A, respectively, members of the kinesin super-family of proteins. Thus, kinesins, which have been suspected of being potentially related to both asbestos exposure and altered cell division in cancer, may be useful new biomarkers of carcinogenic risk in asbestos-exposed cohorts.

At the same time, parallel to these biomarker studies, we initiated the development of new interventions designed to target the specific molecular defects identified by the biomarker studies, focusing first on p53-related interventions based on the initial results with the p53 autoantibody biomarker. Previous studies had demonstrated that the C-terminal region of p53 is a regulatory effector domain for the protein critical to the control of its function. We therefore synthesized a number of peptide sequences based on this C-terminal region (amino acid residues 353-393), as well as other regions, to examine their ability to inhibit cell growth and induce apoptosis in various normal and tumor cell lines (including lung cancer cells as could be induced by asbestos exposure). The peptides were delivered to the cell lines either as peptides linked to the 17 amino acid sequence of the *Antennapedia* homeobox domain to facilitate transmembrane cellular uptake or as plasmid-based mini-genes for the peptide sequences in adenovirus vectors for cellular transfection. The peptides were found to induce apoptosis in tumor cell lines with p53 aberrations (either mutations or over-expression) at micro-molar levels but to have virtually no effect on cell lines that were p53 null or contained normal levels of wild-type p53 (including highly sensitive normal bone marrow stem cells). Since one of the goals of this research was to develop a peptide-based prophylactic to treat individuals at high risk for the development of p53-related cancers before they manifest overt malignancy, the peptides were also tested against pre-malignant cell lines with normal levels of wild-type p53 or with p53 mutations and were again found to selectively induce apoptosis in the latter. Investigation of the mechanism of action of the peptides suggested that the peptide was binding to p53 and restoring its active structure and apoptotic function by inducing increased membrane activity of Fas/Fas ligand and triggering the FADD/FLICE/caspase 8/caspase 3 cascade. Thus, these C-terminal p53 peptides have been successfully demonstrated to activate normal p53 function and be selectively cytotoxic to malignant and pre-malignant cells with altered p53 in cell culture. In order to demonstrate an *in vivo* effect, the peptides were applied to an existing rat brain cancer model based on a mutant p53 rat glioma cell line treated by direct intracerebral infusion of the peptide, which resulted in 52% apoptosis in the tumors of the peptide-treated animals compared to 3% in the controls. To demonstrate the applicability to human cancers, the peptides were next applied to animal models of nude mice xenografted with human cancer cell lines, including the human H1299 lung cancer line expressing either mutant, wild-type or null p53, and shown to be selectively cytotoxic for mutant p53 tumors with substantial improvements in survival and little or no toxicity. These results suggest that p53 C-terminal peptides could be useful for the treatment (and possibly prevention) of p53-related cancers in humans, including many of the types of tumors induced by asbestos exposure. The most effective of these peptides for its cytotoxic effects has been found to be a construct based on a palindromic tetramer sequence of the C-terminal regulatory region (which presumably mimics the normal tetramer structure of wild-type p53), and a patent on this peptide has recently been issued (U.S. Patent No. 7,772,367). Direct human studies with this peptide are currently being planned.

In conclusion, this research has demonstrated that biomarkers (including those based on p53 autoantibodies or kinesin proteins) can be used to identify those asbestos workers who are the greatest risk of developing cancer so that they can then be targeted for more aggressive interventions to prevent or treat their cancer. Specific interventions can be targeted to the exact genetic defects identified by each particular biomarker. For example, individuals with p53 autoantibodies could benefit from new approaches based on the restoration of p53 function by p53 C-terminal peptides that can selectively induce apoptosis of mutant p53 cells *in vitro* and *in vivo.* Finally, when applied in human studies, the progress of the therapeutic or preventive intervention could be monitored by following the biomarker status.

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Inclusion of Gender and Minority Subjects

This research involved Finnish asbestos workers. Due to the nature of this workforce, which historically did not include women or other minorities, very few women and no other minorities could not be included in this study.

Inclusion of Children

No children were not included in this study due to the nature of the populations from which the subjects were drawn, namely adult workers.

Materials Available for Other Investigators

Not Applicable.