Post-translational Protein Modification: Novel Technologies and Implications for Cancer Prevention Workshop

Executive Summary

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Post-translational modifications (PTM) are integral parts of gene regulation. PTMs of proteins, not detected through RNA analysis, may occur at different stages of tumor development indicative of early or late events of transformation. During PTM, a protein is modified due to phosphorylation, acetylation, glycosylation, ubiquitination, farensylation, methylation, sialylation, etc. Specific genes are turned on or off at the onset of initiation, development, and progression of diseases such as cancer. PTMs have been reported for the key steps in tumor progression, such as cell cycle check point, differentiation, and apoptosis. Identifying PTM markers at an early stage of cancer development and developing prevention strategies is a high priority area of research at the Division of Cancer Prevention (DCP), National Cancer Institute (NCI).

To identify the gaps in the knowledge of PTM and their implication in cancer prevention, the DCP organized a 2-day workshop in Bethesda, MD held on August 27-28, 2002. More than 70 scientists participated in the workshop and provided recommendations for future research in this field.

The following topics were discussed during the workshop:

- Applications of technology and clinical questions relevant for cancer prevention, detection, and chemoprevention.
- Prioritization of the technologies suitable for clinical use.
- Identification of PTMs that are the most suitable for development as molecular and surrogate markers.
- Identification of the clinical correlates for the use of PTMs that can be transferred from basic research and technology development and used as tools for early detection, chemoprevention, and risk assessment.

A brief description of the topics discussed and recommendations made during the workshop are given below.

Signal Transduction and Post-Translational Modifications

Signal transduction, via growth factors, proteins, and peptides, plays a major role in cell transformation. Signal transduction pathways may lead to carcinogenesis, involving a monogenic event, which may be inactivation of a tumor suppressor gene, activation of an oncogene, or autocrine growth factor regulation. For example, the inactivation of *pRb* and *p53* may start with an oncogenic event in ras, myc, or other genes. Of two distinct pathways that contribute to cell-cycle control (*$p16^{INK4a}$/pRb/cyclin D1* and

p19^{ARF}/p53/MDM2), at least one is universally inactivated in tumors. Dr. Richard Pestell reviewed the role of cyclin-dependent kinases (CDK) in signal transduction, acetylation and its role in hormone signaling pathways, deacetylation involved with methyltransferases (MTs), the regulation of *HIF1*, the role of hinge/ligand binding domain, and *PIN1*.

PTMs that may contribute to tumorigenesis include phosphorylation, acetylation, methylation, glycosylation, prolyl isomerization, hydroxylation, oxidation, glutathionylation, and ubiquitination. Phosphorylation pathways that are induced by receptor amplification (ErbB2, MET) and activating mutations and rearrangements (*Bcr-Abl*) in turn activate phosphorylation of cell-cycle checkpoint proteins.

Phosphorylation by the cyclin-dependent kinases inactivates tumor suppressors of the pRb family to promote cell-cycle progression and contact-independent growth. Acetylation/deacetylation of diverse substrates (histones, transcription factors, p53, androgen receptor, microtubule proteins) regulates metabolic function and cellular growth. Trichostatin A (TSA) sensitive (class I and II histone deacetylases [HDACs]) and NAD-sensitive (silent information regulator 2-Sir2) HDACs regulate cell proliferation and senescence. Rearrangements of histone deacetylation recruitment complexes (i.e., PML-RAR, PLZF-RAR) are key genetic events in leukemogenesis and HDAC inhibitors block tumor growth. HDAC complexes may regulate aberrant growth through multiple mechanisms. For example, Sir2 binds and deacetylates p53 to inhibit its transcriptional activity and block senescence induction. Arginine methyl transferases augment activity of hormone receptors and promote expression of cell-cycle control proteins. Oncogenic in some cases of T-ALL, hNOTCH, is modified by Fringe glycosyl transferases, which in turn alters Notch ligand specificity and activity. The von Hippel-Lindau (VHL tumor suppressor) ubiquitin ligase binds and regulates HIF1 function in an oxygen-dependent manner. Full activity of HIF1a involves both proline and asparagine hydroxylation (by oxoglutarate-dependent dioxygenases). The E3 ligases regulate ubiquitination and protein stability. Dysregulation of tumor suppressor degradation may contribute to tumor growth. The SCF E3 protein p45^{Skp2} for example, which targets degradation of the CDK inhibitor p27^{Kip1}, can function as a cooperative oncogene.

One of the PTMs is ubiquitination of proteins resulting in degradation of proteins. Ubiquitinin is a highly conserved 76 amino acid-long protein. It functions by binding to another protein and modifying the substrate protein. Depending on a specific reaction and requirement of the cell, either one or several ubiquitinin molecules are attached to the substrate protein. If a single ubiquitinin attaches to the protein, it is called monoubiquitination, which is generally required for the internalization of a protein, such as receptor mediated internalization. On the other hand, several ubiquitinin molecules may bind (polyubiquitination) to a substrate protein that result in degradation of proteins (sometimes mediated by proteasomes).

Compared to histone acetylation, histone ubiquitination has not been studied extensively. Ubiquitination of histones H1, H3, H2A, and H2B has been reported *in vivo* although the most prevalent ubiqutination occurs in uH2a and uH2B. One of the widely studied proteins that undergoes ubiquitination for its activity is p53. Among several functions that p53 protein performs, one is DNA repair. Inactivation of p53 results in loss of this function and induction of apoptosis. The level of p53 in a cell at any point thus reflects a "life and death" situation for a cell. Inactivation of p53 is caused by degradation via ubiquitination of p53. Ubiquitination is a PTM where ubiquitinin binds to p53 and induces a cascade of events as a part of the ubiquitinin degradation pathway. The major enzyme that participates in this event is Mdm2.

Monoubiquitinated proteins are stable, whereas polyubiquitinated proteins are transient and susceptible to degrade fast. In the whole process of degradation, a cascade of enzymes participate. For example, E1 enzyme is needed for activation, and conjugase and ligase are required for the degradation of the polyubiquitinated protein. Binding of ubiquitinin occurs via lysine residue on the substrate protein. How substrate proteins are chosen for monoubiquitination and polyubiquitination is not completely understood. When a cell needs to reduce the load of ubiquitinin, it can remove an ubiquitin molecule by an enzyme activity and deubiquitination occurs. Thus, the cellular pool of ubiquitinin is maintained in a highly organized way.

Post-Translational Modifications in Histones

In eukaryotic cells DNA is associated with proteins to form a complex known as chromatin. The most fundamental unit of chromatin and the first level of DNA organization are nucleosome core particles. Each nucleosome consists of 146 bp DNA wrapped around the core octamer histone. The negative charge of the nucleic acid is thus neutralized with the basic histone proteins resulting in stabilization of the chromatin. The dominant proteins within this chromatin complex are the histones, which are subject to a wide variety of covalent and reversible post-translational modifications such as acetylation, ubiquitination, and farensylation. Although it is important to know what changes occur during PTM, it also isessential to know when these changes occur and what the factors that induce PTM are.

Histone Acetylation

A specialized family of enzymes, the histone acetyl transferases, catalyzes the transfer of acetyl groups from their cosubstrate acetyl-coenzyme A to lysine residues of histones. Acetylation of histone N-terminal lysine residues induces chromosomal changes and results in the loss of chromosomal repression that allows the successful transcription of the underlying genes. Analogously, in DNA repair and also DNA replication the chromosomal repression is thought to be relieved by such mechanisms. Thus histone PTMs are essential for chromatin structure and dynamics. Most of the PTM in histone occurs in the tail region as histone tails protrude out from the nucleosome and are exposed to modifications. Currently, histone tail PTMs are considered to be the most critical steps in chromatin dynamics and conformation.

Dr. Paul Marks presented information on histone deacetylase (HDAC) inhibition and described investigations on modifying the histone protein structure by affecting the state of acetylation or deacetylation and altering gene expression. Nuclear histone acetylation is a reversible process and is regulated by a group of histone acetyltransferases (HATs), which promote acetylation, and HDACs, which promote deacetylation. There are 10 or 11 HDACs, which are classified by homology as class I or class II. Class II HDACs are much larger molecules than class I HDACs. In addition, class III HDACs has been identified that are dependent on N-acetyl cysteine (NAC) for its deacetylase activity and are not inhibited by compounds that inhibit class I or class II HDACs.

A number of inhibitors of HDACs are known that are in either phase I or phase II trials, including valproic acid, suberoylanilide hydroxamic acid (SAHA), pyroxamide, trapoxin, FK-288 (depsipeptide), MS-275, and N-acetyl dinaline (CI-994). Dr. Marks illustrated the promise of HDAC inhibitors in clinical trials investigating the hydroxamic acid-based HDACs SAHA and pyroxamide, which are potent HDAC inhibitors that cause growth arrest, differentiation, and/or apoptosis of many tumor types *in vitro* and *in vivo*. The mechanisms-of-action of inhibition of deacetylation and the manner in which they allow the accumulation of acetylated histones in the region of genes that lead to increased

transcription are not very well elucidated and are topics for future research. HDAC inhibitors have been shown to be selective for transformed cells and they are very selective for specific genes that have altered transcription. For example, *p21*, an inhibitor of cyclin CDK, is associated with the arrest of cells in G1 and is markedly increased in transcription rate and the accumulation of its protein.

Dr. Marks discussed the example of TBP-2, which becomes inactivated due to deacetylation. Treatment with the HDAC inhibitor SAHA resulted in increased transcription of *TBP-2*. In addition, with the induction of *TBP-2*, there was a decrease in reduced thioredoxin, which is required for transcription. In cancer and normal cells, *TBP-2* is expressed at normal or low levels in colon or breast cancer cells.

HDACs have been shown to increase cell differentiation and apoptosis in a variety of tumor cell lines, including murine erythroleukemia, human bladder carcinoma, neuroblastoma, myeloma, breast adenocarcinoma, and prostate carcinoma. Animal studies report that HDACs have been effective in *in vivo* models for prostate cancer, leukemia, neuroblastoma, breast adenocarcinoma, and lung tumors.

Histone Methylation

Diet can alter gene expression, and the methyl donor in diet may cause inactivation of a number of genes via hypermethylation (epigenetics). Dr. Shi Huang presented results on diet and cancer and on the role of histone methyltransferases (MTs) in carcinogenesis. Diet, especially the high intake of red meat and fat and the insufficient intake of vegetables and fruit may lead to cancer. Understanding the role of nutrients in preventing or causing cancer is a challenge for epidemiologists, molecular biologists, and cancer researchers. Dr. Huang focused on methyl donors as an important area of study for understanding the effect of diet on cancer. The first suggestion of a link between methyl groups and cancer was the discovery of tumor suppressor genes that had MTs, which require a methyl donor as a cofactor. Methyl donors can be supplied by diet.

Epidemiological and clinical studies indicate that a decreased SAM:SAH ratio is a common theme in the development and progression of cancer. Possible mechanisms include the following:

DNA Methylation. This concept is controversial because hypermethylation of tumor suppressor genes is important for increasing the carcinogenesis process. If the diet is deficient in methyl groups, there does not appear to be a connection between the deficiency and increased hypermethylation of these genes.

DNA Mutation. This concept involves the deficiency of folic acid, which leads to an increase in incorporation of UTP to DNA and DNA mutations.

Histone Methylation. In this scenario, 5,10-methylenetetrahydrofolate (MTHFR) polymorphisms may account for higher levels of homocysteine and SAH, which leads to lower MT activity.

Implications for cancer prevention include increasing the intake of folic acid by intake of foods such as whole grains, asparagus, beans, broccoli, liver, fish, and eggs. Dr. Huang suggested that the clinical goal for treatment recommendations should be to modify the SAM:SAH ratio, target individual enzyme-targeted SAM donors, target specific enzyme modulators of MTs, and investigate applications for gene replacement therapy. Future research may include developing improved animal models, investigating how diet affects histone methylation, and the role of altered histone methylation in carcinogenesis.

Research also must answer questions about the specific mechanisms that inhibit proliferation and improve differentiation and chromatin structure, and about the interaction with known genetic pathways of cancer (e.g., *Rb*, *p53*).

Technologies to Detect PTM and Utilization of PTM as a Tool for Detecting Cancer

Laboratory-based techniques for detecting the molecular and genetic changes are often hindered by expense and time. To be an effective screening tool at the population level, automation and cost effectiveness have to be built into these technologies. In addition, minute amounts of a biomarker should be detected with high precision in terms of specificity and sensitivity. Recent advances in genomics and proteomics (especially functional proteomics) hold great potential for diagnostic, prognostic, and therapeutic applications.

Proteomics encompasses in its definition, methodologies directed towards the detection of the functional status of a cell through "proteome" analysis. The cell's proteome is the protein counterpart of the genome, and hence, proteomics deals with functional units of expressed genes. Proteomics is valuable in biomarker discovery as the proteome reflects both the intrinsic genetic program of the cell and the impact of its immediate environment. Proteome analysis is enabled largely through biochemical analysis of cellular proteins to provide a protein fingerprint. Distinct changes occur at the protein level during the transformation of a normal cell into a neoplastic cell, which includes altered expression, differential protein modification (PTM), changes in specific activity and inept localization, all of which may affect cellular function.

PTMs of proteins, not detected through RNA analysis, may occur at different stages of tumor development indicative of early or late events of transformation. Recent advances in mass spectrometry have helped complement traditional protein chemistry methodologies in detection and identification of proteins. Through the use of matrix assisted laser desorption/ionization (MALDI) and tandem mass spectroscopy (MS), mass spectral analysis of proteins is helping in their rapid detection, identification, and sequence analysis from biological fluids. Protein-chip technology using chips with surface enhanced laser desorption/ionization (SELDI) is being developed as a high-throughput assay for a panel of markers in establishing protein fingerprints. Immobilizing specific antibodies on the chips allows for quantitation of distinct proteins from different samples or tumor stages. By providing protein fingerprints, proteomics contributes towards early detection and diagnosis of cancer in addition to providing important information on therapeutic targets, and in monitoring effects of pharmacologic agents. Changes in protein expression patterns in different epithelial tumors, such as decreases in tropomyosin and cytokeratin expression and increases in proliferating cell nuclear antigen (PCNA), have been reported. Similar to genomics, output data from proteomic applications are being interpreted after computer processing. Chip technology that can characterize proteins, from complex biological fluids at subfemtomole levels, by combining biological interaction analysis and mass spectroscopy, holds great promise in biomarker discovery.

Incorporation of technologies such as proteomics into biomarker research holds promise for early detection in providing the patient with a survival advantage. Analysis of stage-specific tumors, healthy tissue adjacent to the tumor and specific cell populations within the tumor, will help illuminate molecular networks and mechanisms involved in pathogenesis. Problems such as patient compliance, low sensitivity, and specificity affect current investigative procedures such as endoscopic examinations and radiologic procedures. Additionally, the diagnosis of lesions in asymptomatic patients such as

ductal carcinoma in situ, found during mammography, or Barrett's epithelium found during an upper gastrointestinal endoscopy, pose serious questions about hazardous interventions because it is not possible to predict if the lesion will progress to invasive cancer. Molecular signatures obtained through high-throughput technologies, which allow for the concurrent analysis of hundreds of genes and proteins, could help diagnose premalignant lesions. This would provide a means for screening and surveillance to identify high-risk individuals and warrant definitive treatment in individuals who have molecular but not pathological or microscopic features associated with frank malignancy. Nominal use of invasive procedures with fewer complications would be necessary, as surgical management of the potential cancer would be performed at a stage prior to invasion and metastases.

The high-throughput technologies provide a means of classifying disease heterogeneity, under apparently clinically identical conditions through analysis of molecular signature patterns, such as that seen in B-cell lymphoma. Investigating the molecular patterns may provide a measure of the tumor aggressiveness, help predict patient outcome, and augment current staging classifications. They also could provide a means for monitoring progress in response to therapy. Subtle differences in individual profiles of molecular signatures could help physicians to predict how a cancer will respond to specific chemotherapeutic agents, and treatments tailored to the individual patient could be designed. Molecular information on disease susceptibility could help in the development of individualized medicine useful in prevention, early detection, and drug responsiveness in choice of treatment.

Dr. Hanash presented information on how PTMs are emerging in his studies as the major source of cancer biomarkers. A majority of the biological changes in diseases such as cancer and diabetes occur in the proteome rather than in the genome. It is important to know where the protein is located; not just what changes have occurred. Dr. Hanash illustrated the difference between a genomic or microarray approach and a proteomic approach for cancer. Dr. Hanash reported that he had investigated the gene *Op18*, which was highly expressed in leukemia. With DNA microarrays, it is possible to determine whether *Op18* is expressed or not expressed; however, at the protein level, it is possible to determine that there are 11 different forms of *Op18*, all of which are due to PTMs. Investigating *Op18* by proteomics allowed identification of the specific oncogenic mutation in one form of *Op18*. This level of analysis is not possible with DNA microarray studies.

The contribution of PTMs to the detection and identification of cancer biomarkers is significant. One approach is to rely on the immune system to help identify proteins that are synthesized by tumor cells that may be immunogenic. It may be possible to detect antibodies against the proteins that can be used as biomarkers. This is a simple approach that can be very productive. Dr. Hanash listed three examples of proteins that are immunogenic in cancer-beta-tubulin3 in neuroblastoma, annexin I and II in lung cancer, and calreticulin in liver cancer-that have been investigated to determine whether they can be used for early detection of cancer. For example, there appears to be some promise for using antibodies to annexin in the early detection of lung cancer. Studies on serum from patients in the Carotene and Retinol Efficacy Trial (CARET) trial indicated that 3 of 10 patients who progressed to lung cancer had antibodies to annexin 1 year before diagnosis.

Dr. Hanash described the use of liquid electrophoresis to resolve the proteome in tumors and tumor cell lines. This is multidimensional protein separation, which has much better resolution than anion exchange chromatograms. Liquid Chromatograph (LC)-Tandem

Mass Spectrometer (TMS) is an MS-MS instrument combined with LC to measure molecular species disassociated from a matrix, and allows determination of sequences of peptides.

MS is used to image and profile proteins from tissue sections. It can be used in proteomics for a variety of chemistry investigations, including determining primary structure and higher order structure, PTMs, protein-ligand and protein-protein interactions, quantitation, and image analysis. There are two main MS technologies for protein studies:

In Matrix-Assisted Laser Desorption/Ionization (MALDI)-Time-of-Flight (TOF) MS, samples are irradiated after coating the sample with an energy-absorbing matrix. Molecules from the surface are dissolved and accelerated, and their TOF is measured to determine molecular weight, mass, and electrical charge.

Dr. Caprioli described examples of the use of MALDI-TOF and LC-TMS technologies to develop images from protein samples. Images developed from these methods can illustrate the distribution of a specific molecular weight molecule (i.e., protein) in a tissue slice. The range of molecular weights using these methods is between 1,000 and 100,000 m/z. The MALDI-TOF results are very reproducible. The profiles produced by these methods may be used to follow the progression of disease, but not to classify the disease. Dr. Caprioli expressed the opinion that these methods can identify protein patterns to be used as biomarkers or can be used to determine the efficacy of drug therapy with biopsy material. A finding that may be applicable to both areas is that different proteins exist in different areas of a tumor. Using the example of mouse epididymis, Dr. Caprioli showed that different proteins exist in tissue along the length of the epididymus.

Another improved technology, MALDI-QSTAR (QqTOF) is used to determine concentration of chemoprevention agent in tissue. The QSTAR is a quadrapole TOF MS-MS machine and can be very specific for very low concentrations of the agent. This technology can help show the positive correlation between LCMS analysis, which can take days to analyze, and MALDI imaging, which can be completed within minutes.

There are several advantages of the MALDI technology: it is a high throughput discovery tool, can perform direct analysis of tissues, has high molecular specificity, can be used for simple protocols, and needs a small amount of sample and result in multiple images of different molecular weights. These technologies can be used for protein identification and in the study of PTMs. The highest sensitivity of MALDI is in the molecular weight range below 50 kDa. Although it currently is possible to analyze proteins up to molecular weight 30 KDa, there is a decline in accuracy and specificity above 50 kDa. There is a requirement of further processing for protein identification.

Benjamin Cravatt discussed activity-based protein profiling. The goal of this research is to develop technologies, primarily activity-based chemical probes (ABPs), to measure protein function directly in complex proteomes. Regarding enzymatic activity and the role of PTMs in transformation, there are three significant enzyme types that have become the focus of research in this area: proteases (zymogens, endogenous inhibitors), kinases (autoinhibitory domains), and phosphatases (autoinhibitory domains, endogenous inhibitors).

The challenge of activity-based protein profiling is identifying active sites on specific enzymes, which guide their chemical activity, and finding chemical reactive groups that label and bind the active sites in complex proteomes. The first enzyme class targeted

with this approach was the serine hydrolases (SHs), a large and diverse enzyme family involved in a variety of pathophysiologic processes, including blood clotting, angiogenesis, neural plasticity, peptide hormone processing, T-cell immunity, reproduction, cancer, emphysema, and plant-parasite interactions.

Inhibition of SHs can be achieved by the ABP fluorophosphonate (FP) reagents. A study of breast and melanoma cancer cell lines indicated that it is possible using cluster analysis to characterize cell lines based on subcellular distribution of enzyme and tumor of origin. In this study, one cluster each of breast and melanoma cells was identifiable. A third cluster formed that had one breast line, one melanoma line, and one line of unknown origin and shared the property of being invasive. This suggested that cell lines may cluster by their invasive properties rather than by their tumor of origin. Thus, a dedifferentiation phenotype cell line model was established.

Dr. Cravatt described enzyme-specific information derived from the investigation of the breast and melanoma cell lines. Sialyl acetylesterase (SAE), which is involved in PTM, actively reacted with the APB, although it bears no sequence homology to other proteins. Another noticeable finding was that invasiveness-related serine hydrolase (IRSH), an integral membrane enzyme upregulated in invasive cancer cells from several tumor types, is upregulated in invasive cancer lines independent of tumor origin. Because a correlation of message expression with activity does not exist, some proteins that are regulated post-translationally have a message level different from activity level, which is indicative of post-translational regulation. Further research is necessary to investigate whether any of the SHs upregulated in invasive cancers are required for their aggressive properties. In summary, a global analysis of SH activities provides proteomic information of sufficient quantity and quality to permit the phenotypic classification of cancer cells; invasive and noninvasive cancers exhibit nearly orthogonal serine hydrolase activity profiles; secreted and membrane enzymes are better predictors of cancer phenotype than cytosolic enzymes.

Applications of PTM Technologies to Clinical Chemoprevention Research

Dr. Lance Liotta presented surface enhanced laser desorption ionization time-of-flight-mass spectrometry (SELDI-TOF-MS) results for discovering proteomic patterns in blood that may offer a novel approach for early cancer detection. Proteomic signatures emanate from the cell as cancer develops and the emanating proteins generate biomarker profiles that end up in the effluent blood surrounding the cells. The concept is that the collection of proteins flowing into the tissue can be modified as it leaves the tissue due to enzymatic events from the cancer cell or the host, or proteins could be added to the perfusing blood as biomarkers. The serum proteome is a complex of thousands of proteins and peptides and the smaller post-translationally modified proteins that are cleaved, glycosylated, phosphorylated, or tagged with a lipid, provide a wealth of diagnostic information about the pathologic state of this tissue.

In the SELDI pattern, the diagnostic proteins are identified without knowing their function. One challenge is to identify the proteins derived in the cancer cell from those derived from the host cell. This is a low resolution system that can process one drop of raw serum on a microchip that binds proteins. A profile of differentially expressed proteins is generated. After validating the protein patterns, an algorithm was developed to find the subset of proteins that are diagnostic of the cancer compared to the noncancer state. The genetic algorithm takes two sets of training data, shifts through them, and finds the subset pattern that can tell the difference between the two training populations. That pattern is mapped in n-dimensional space as a series of clusters and

an unknown sample can be compared to the cluster to determine if it is a cancer or a normal pattern.

SELDI analysis of serum from prostate and ovarian cancer patients and a comparison with normal serum identified distinct disease specific protein profiling. Dr. Liotta presented clinical results from patients with ovarian and prostate cancer. Results have been encouraging for identifying cancer in men with prostate specific antigen (PSA) levels between 4 and 10 mg/ml, which is a level that has no specificity for that test. The use of the multiparametric proteomic patterns application method was able to identify men that subsequently developed prostate cancer. Results of investigations for ovarian cancer showed that the algorithm could identify patterns that were subsequently found to be cancer. They achieved very high specificity (95 percent) and sensitivity (100 percent) in this study.

The hope is that if cancer researchers can shift the diagnosis of ovarian cancer from stage III, where it currently exists, to stage I, many lives will be saved. Moving to a clinical testing situation will demand a more advanced and reliable system than what is now available. Therefore, current efforts are being made to move from the SELDI-TOF-MS to the QSTAR technology, which can offer much higher resolution, quicker response times, and assessment of a wider molecular weight range of proteins. In preliminary tests with the QSTAR, results have been very encouraging.

Regarding cancer prevention, the currently accepted definition of chemoprevention is "intervention with pharmaceuticals, vitamins, nutrients, and minerals, or other chemicals that reduce cancer risk." Dr. Brenner suggested that choosing the targets for chemoprevention research is of the utmost importance. Common, lethal tumors for breast, lung, colon, and prostate cancers should be targeted because these four cancers have a large impact on the population. There also should be evidence of a nongenetic component (i.e., acquired mutations and epigenetic changes) to the carcinogenesis process in the target disease. Another important issue is the ability to obtain tissue to study the disease, and there must be some amount of preclinical (e.g., mechanism-of-action, *in vitro* studies, and preclinical *in vivo* data) evidence to support the research.

A number of limitations exist in chemoprevention trials; for example, the amount of effort that is needed to recruit and accrue participants to chemoprevention trials. Some studies indicate a screen-to-accrual rate of 5-10 percent. A significant barrier is the number of participants needed to reach a cancer endpoint to a trial. Large numbers of participants will be needed for a significant period of time (10-20 years) if cancer is used as an endpoint. In addition, the design of chemoprevention trials is very complex.

Utilization of surrogate endpoint biomarkers (SEBs) addresses many of the limitations seen in large chemoprevention trials. However, there always will be a question of whether the surrogate endpoint is a valid step along the pathway to carcinogenesis. Three types of SEBs that are being used in chemoprevention research are: (i) *Pathologic Biomarkers* are premalignancies, such as colon polyps, that are known to progress to cancer in some cases; (ii) *Drug Effect Biomarkers* are dynamic endpoints of a direct drug-to-target effect, such as aspirin, which inhibits the production of prostaglandins; and (iii) *Biological Biomarkers* such as proteins, genes, or quantitative cellular processes are used to predict cancer diagnosis or risk. These can be divided into *Process Biomarkers*, such as those involved in pathway-derived carcinogenesis or regulatory endpoints, and *Screening Biomarkers*, which are derived from proteomic assessment.

It is known that redundancy exists in signal transduction pathways and redundancy often leads to confusion about the effectiveness of an intervention. It is difficult to determine whether the effect seen by an intervention has been successful, even if the biomarker is altered and there is a reduction in the tumor. It is important to quantify the effect of the intervention on the biomarker, especially in phase I trials, where there are no clinical endpoints.

For biomarker validation, information about quantifying biomarker endpoints is essential. There should be strict laboratory standards and procedures for completing assays. Validation is very difficult unless newer technologies are used to increase statistical power. There is a need for reliable biomarker panels for use in large population studies that meet the following specifications: (i) serum is the preferred sample for the assay; (ii) the assay should not be expensive; (iii) there must be an easy method for quality control; and (iv) there must be an algorithm because proteins, which are multiclonal, will vary among different types of cancer.

To achieve above goals, numerous biomarkers should be investigated to rapidly determine their usefulness, to characterize biomarkers in a cross-sectional study so that there is lower percent prevalence of false positives and false negatives. Biomarkers should be assessed in one panel.

Targetable Post-Translational Modifications

Kinases appear to play a key role in carcinogenesis, as the majority of the oncogenes and proto-oncogenes involved in human cancers code for protein tyrosine kinase (PTKs). PTKs have been linked to human disease for the past 15 years, and more than 500 different kinases have been identified in the human genome. Jeffrey Hanke presented information on PTK inhibition and an update on IRESSA (ZD 1839), a novel therapeutic compound. The epidermal growth factor receptor (EGFR), which is the target of IRESSA, also has been a target for cancer researchers for 4 decades. EGFR is overexpressed in a number of tumor types and appears to be a good prognostic indicator of these tumors. In all cells that express EGFR, IRESSA inhibits autophosphorylation *in vitro*.

IRESSA acts on various cancer cell processes, such as tumor growth, invasion and metastasis, angiogenesis, and apoptosis. The multiple effects may be due to IRESSA's action through EGRF and other receptor tyrosine kinases, which signal through multiple pathways that influence various mechanisms. For example, one of the principle EGFR signaling routes is through the MAP-kinase pathway that can crosstalk to other growth pathways, such as estrogen receptor (ER), *bcl2* and *bax*, and EGFR. The endpoint of IRESSA action is an upstream, proximal target marker, which is not adequate to predict tumor growth.

In preclinical models, it has been shown that IRESSA can slow growth in tumors that express EGFR; when IRESSA is removed; the tumor begins to grow at a normal rate. In phase I trials, IRESSA was shown to be very effective in non-small cell lung cancer (NSCL). Phase II and III trials currently are underway, with promising results in non-small cell lung carcinoma (NSCL) patients.

There remains some unanswered questions that must be answered regarding PTKs, including: (i) PTKs can be safe and effective for cancer treatment, but the question remains whether they should be used as monotherapy or in therapeutic combinations; (ii) it will be important to be able to preselect responsive tumors; EGFR possibly is not the only target that can predict positive action by PTKs; (iii) further research is needed to

determine whether *k-ras* affects responsiveness; and (iv) there is a need for early markers of response.

The challenge involved in developing drugs for cancer prevention still exists, better models need to be developed for disease progression, and decisions must be made regarding commercial and regulatory issues.

Another example of signal transduction intermediate targeted intervention is integrin-linked kinase (ILK), a protein kinase that can activate some of the same pathways described for PTK. The focus has been on cell adhesion and anchorage-independent cell growth, which are important properties of tumor cells because they allow the cells to avoid apoptosis. Dr. Sahukat Deodhar described the interaction of the cell with the extracellular matrix and the role of integrins in mediating these interactions and in providing signals to the cell related to cell growth and health. ILK is involved in phosphorylation; inhibitors have been developed that are specific for this kinase.

ILK growth signaling properties are involved in connecting integrins and growth factors to downstream effector proteins, such as protein kinase B (PKB)/AKT, GSK-3, myosin light chain (MLC), and myosin phosphatase. By inhibiting ILK activity, there is a suppression of phosphorylation of PKB on serine-473. This, in turn, causes a suppression of apoptosis. This is only one of the downstream pathways that are affected by ILK inhibition. The ILK can regulate the phosphorylation of PKB/AKT on serine-473. ILK also controls the expression of cyclin-D1; inhibition of ILK inhibits the expression on cyclin-D1 in prostate cancer cell line. Additional experiments have shown that ILK affects: the PKB pathway, resulting in increased cell survival; the beta-catenin pathway resulting in the activation of cyclin-D1; and the AP1pathway, resulting in increased expression of matrix metalloproteinase (MMP)-9. Experiments on colonic polyposis, which is an early stage in the development of colon carcinoma, have shown that ILK activity is higher in polyps than in normal tissue. ILK also is more highly expressed in colon adenocarcinoma than in controls, and ILK levels also are elevated in these tumors. It also has been reported that ILK expression increases in prostate tumor grade, which shows that ILK expression may be a marker for tumor progression as well as for initiation. Additionally, in a rat orthotopic lung cancer model, an ILK inhibitor, alone or in combination with cisplatin, dramatically increased animal survival.

ILK may be a good therapeutic target for the treatment of early cancers, as well as during cancer progression, because it exists at the nexus of signaling pathways between cell adhesion molecules and receptor tyrosine kinases, and its activation is required for survival through extracellular matrix interactions. When ILK is disregulated, it can activate downstream proteins such as PKB/AKT, the beta catenin pathway, or the AP1 pathway.

Recommendations and Future Research Directions

The following recommendations were made during the workshop:

1. Technology advancement is needed for high-throughput quantitative assays to determine PTMs.

A decision should be made as to whether PTM investigation should proceed as a "backward approach," moving from cancer to precancerous lesions, or a "forward approach," which would involve a comprehensive analysis of PTMs in early lesions to determine which are predictive of future cancer. Another issue that must be considered is how to approach conducting a comprehensive analysis of PTMs in cancer-related genes and pathways and which genes and pathways to focus on initially.

Areas of challenge included PTMs of proteins and how to prioritize these events based on their use for early detection, risk assessment, and chemoprevention; protein compartments; ligands/antibodies; early/preneoplastic lesions; biomarkers/fluids; integrating protein data; and links to metabolism/nutrition. The focus should be on prevention, although using techniques commonly associated with treatment should be considered.

Technology and Resources Issues. Current technologies should be utilized until new technologies are found appropriate for proteomic studies. The case may be that current technologies are underutilized at this time. Creation of a "tool set" for different PTMs would facilitate research. The focus of developing technology should address how to best investigate the sub-cellular components or protein compartments, and macrocomplexities of protein-protein interactions.

Resources Needed. Development of ligands and antibodies for PTMs should be considered a research priority. There are tissue resources available that could be used in proteomics; an assessment of available tissue resources may be undertaken to determine what exists and how it could be procured. There are very good animal models for human cancer than may be able to be adapted to proteomics. Issues of database development and quality control should be addressed before proteomic research moves further ahead for clinical applications.

Integration of Protein Data. Some thought must be put into the most appropriate manner for integrating clinical and genomic data with proteomic data.

The specific recommendations to address the issue of proteomics and technology included the following:

Create Centers for Cancer Proteomics. Research areas to be included in these specialized centers include technology, specialists in technology working hand-in-hand with clinical investigators, biological/specimen/tissue/animal model resources, informatics, and training.

Development of Targeted Funding for PTMs. This will give recognition of the importance of proteomics to cancer research.

Targeted Funding for the Development of Resources. Resources may include antibodies and ligands to assay proteins.

2. Basic research is needed to understand the PTMs and their role in carcinogenesis.

The study of PTMs is in the discovery stage, and there is a need to have centers where the objectives for the clinicians, as well as the basic science, are to capture specimens in a manner that is appropriate to clinical use. The following recommendations also were made:

PTMs are useful as predictive markers and may be used to identify cohorts for clinical studies. Drug effect markers also may be an important tool for drug selection for chemoprevention trials. An example may be to use PTMs as a marker to quantify proliferation in a preneoplastic lesion, rather than waiting for cancer to develop.

There is a need to know if markers exist in tissue and pathways identified in epidemiologic studies, and how these relate to identified risk markers.

For discovery and phase I and II clinical trials, all samples would be appropriate, including serum and other relevant body fluids (e.g., nipple aspirate fluid, urine, and saliva). For phase III trials, serum and other body fluids are appropriate.

Specific recommendations were developed through the discussion of clinical issues related to proteomics, which included the following:

The Discovery Stage for PTMs Should Be Extremely Wide-Reaching. During the discovery phase, there is a need to investigate differential marker patterns and a wide variety of normal, precancerous, and cancerous conditions in the tissue as a whole and in different compartments. In addition, normal and precancerous lesions in areas within the same individual should be investigated to determine if there is a differential marker pattern within those compartments. There also is a need to investigate the same types of lesions across many different individuals.

There is a Need to Develop Standardized Protocols for Sample Collection. Samples for proteomic investigations are not collected in the same manner as samples for other research investigations. This is a tremendous problem for proteomics research and should be addressed before investigation of PTMs proceeds much further.

There is a Need for a Large Prospective Cohort Trial. The trial should be conducted in individuals that are characterized as having low, average, or high risk for the cancer of interest. Assessments should be made over time using invasive and noninvasive methods. Validation should be one of the main components of this trial. Development of a reference standard, such as normal serum, also is required for optimization of results in SELDI analysis.

In summary, the workshop generated new directions for future research in the field of utilization of PTMs in cancer detection and developing chemoprevention strategies.