Seventeenth Annual Pezcoller Symposium: Molecular Understanding of Solid Tumors

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Introduction

This symposium was held June 16 to 18, 2005 in Trento, Italy and was cochaired by Drs. William R. Sellers and Enrico Mihich. Molecular and genetic alterations found in distinct cancers were discussed for their implications in signaling pathway deregulation and identification of novel therapeutic targets. Emphasized were discoveries of cancer dependencies through modern technologies and characterizing individual tumors towards projecting their response to individualized intervention. William Sellers stated that themes of the meetings are identification of genetic alterations in key tumor suppressor and oncogenes and understanding of related molecular pathways. Included among the pathways to be discussed are the phosphatidylinositol 3-kinase (PI3K)/Akt, the Hedgehog, the mitogen-activated protein kinase (MAPK), and the von Hippel-Lindau/hypoxia-inducible factor (VHL/HIF) pathway. Patient selection remains critical for rapid drug development, although elucidating the variables influencing and predicting response remains difficult. Efforts in drug discovery and development together with efforts to understand the genetic and molecular basis of cancer bodes well for not simply controlling cancer but for developing the appropriate combinatorial therapeutic regimens that will eliminate cancers.

Molecular Classification

Michael Stratton discussed systematic sequencing towards uncovering the so-called "driver" mutations in cancer and highlighted the importance of distinguishing these from "passenger" mutations not conferring selective growth advantage. Screened were point mutations and splice junction alterations for 518 protein kinases in 26 breast, 33 lung, 13 testicular, 20 gastric, and 30 colorectal cancers. Many mutations were found in lung, gastric, and colorectal cancers; in lung and breast cancers, no excess of kinase over driver mutations was seen. Cancers derived from surface epithelia with high turnover, exposed to exogenous mutagens, may accumulate somatic point mutations compared with other epithelial organs; driver mutations might be dispersed among cancer genes, each mutated infrequently. The presence of potential targets in a small proportion of human cancers presents a challenge for targeted therapeutics development.

William Sellers identified large-scale cancer somatic mutations using single nucleotide polymorphism (SNP) arrays and small-scale mutations by primary human tumor DNA exon resequencing. Using high-density SNP arrays, alterations across the NCI 60 cell lines panel were identified; integration of genetic maps with gene expression signatures identified *MITF* as a melanoma amplification target associated with metastatic disease and decreased survival; *MITF* and *BRAFV600E* transformed immortalized human melanocytes. *MITF* is a "lineage survival" oncogene required for tissue-specific development and tumor progression. Targeting MITF alone or with BRAF may offer new therapeutic approaches for melanoma.

Joseph Nevins discussed the application of gene- and pathway-specific expression profiles to develop predictive signatures for patient stratification. Non–small cell lung carcinoma (NSCLC) stage IA requires surgery, whereas stages IB, II, IIIA require chemotherapy. Samples classified clinically as stage IA were in fact stage II or III based on integrative genetic expression signatures. Signatures for oncogenic signaling pathways, including Ras, Myc, Src, β-catenin, and Rb-E2F pathways, were identified. These functional signatures were used to predict pathway activity in breast, ovarian, and lung cancers. Predicted pathway deregulation and sensitivity to pathway inhibitors were related in breast cancer cell lines. Combining risk stratification or chemotherapy response prediction with pathway analysis may allow designing effective treatments for advanced cancer.

Giovanni Tonon used comparative genomic hybridization (CGH) array and expression profiling for cancer gene discovery. Primary NSCLC (18 adenocarcinomas and 26 squamous cell carcinomas) and 34 NSCLC cell lines were analyzed using oligonucleotides or cDNA arrays. These profiles revealed rearranged NSCLC genomes and allowed definition of 74 amplifications and 19 deletions with 1.53 Mb median size. Twenty-one of 93 regions were <0.5 Mb and spanned a median of only five genes. CGH array data from lung and pancreatic adenocarcinomas were compared: 20 novel shared loci and one shared amplicon contained *ID1, COX4I2, BCL2L1, TPX2*, and *MYLK2. TPX2*, required for spindle formation, was the only gene showing high copy number–driven expression in most lung cell lines and primary tumors. Shared locus of focal amplification included *FGFR1, WHSC1L1*, and *LETM2*. Small interfering RNA (siRNA)–mediated knockdown of *WHSC1L1* resulted in 50% reduction in anchorage-independent growth, whereas FGFR1 depletion had no effect. Using gene-specific CGH, bioinformatic tools, with expression profiles integration identified new amplifications and deletions in the NSCLC genome.

Targets for Sensitivity and Signaling

Hugues de The discussed acute promyelocytic leukemia (APL) as a model for oncogene-targeted combination therapy, focusing on all-*trans* retinoic acid (RA) and arsenic trioxide. The former induces differentiation and remission in APL through interaction with the RA receptor (RARA) fusion with promyelocytic leukemia, transcriptional activation of promyelocytic leukemia/RARA, and relocalization of promyelocytic leukemia towards nuclear bodies. Arsenic trioxide induces complete remissions in APL through differentiation and induction of apoptosis. Arsenic induces

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promyelocytic leukemia/RARA degradation, restores nuclear bodies, and enhances the proapoptotic effects of promyelocytic leukemia, suggesting that targeting nuclear body–associated protein onto the nuclear matrix sensitizes cells to apoptosis. Arsenic induces matrix targeting of promyelocytic leukemia and promyelocytic leukemia degradation, which occurs after sumoylation and proteasome recruitment: arsenic-resistant APL transgenic mice were developed with a mutation in the promyelocytic leukemia sumoylation site. RA-resistant leukemic cells are arsenic sensitive and vice versa. Combining the two agents eradicated leukemia in animals; in patients, blast clearance was greatly accelerated, and complete remissions were achieved. Cyclic AMP (cAMP) triggers APL cell differentiation, enhances RA- or arsenic-mediated differentiation, and reverses RA resistance due to point mutation in the RARA binding pocket of promyelocytic leukemia/RARA. RA plus cAMP activates/derepresses promyelocytic leukemia/RARA–dependent transcription in RA-resistant leukemia. Agents stabilizing endogenous cAMP have clinical value in RA-resistant APL patients. Thus, the promyelocytic leukemia/RARA oncogene can be targeted by three different agents, leading to durable complete remissions in all patients.

Pier Paolo Pandolfi stressed that genes involved in hematologic malignancies are not necessarily specific for these malignancies. Aberrant transcription factors implicated in acute myelogenous leukemia are also involved in the pathogenesis of solid tumors. Nucleophosmin (*NPM*) involved in leukemia and lymphoma pathogenesis can act as an oncogene interfering with tumor suppressor IFN regulatory factor-1 or being a degradation target of ADP ribosylation factor. It regulates p53 through direct binding and hMdm2 inhibition, indicating a potential role in the antiproliferative response to oncogenic insults. NPM is mainly localized in the nucleolus; it participates to rRNA processing and to assembly and transport of preribosomal particles from nucleolus to cytoplasm. NPM localizes to the centrosome in a cell cycle–dependent manner and participates in controlling centrosome duplication. *NPM* hypomorphic mutants were created. $NPM^{-/-}$ are lethal in embryo. p53 induction and concomitant G_1-tetraploid cell cycle arrest were detected in the mutant embryos and in mouse embryonic fibroblasts (MEF). MEFs also displayed mitotic defects with genomic instability and aneuploidy. $NPM^{+/-}$ MEFs are more susceptible to immortalization and transformation, and *NPM* heterozygosity increases Eμ-myc–induced lymphomagenesis. $NPM^{+/-}$ mice exhibit hematopoietic features similar to those of human myelodysplastic syndrome. The genetic region where *NPM* maps to (5q35) can be lost in the "5q syndrome" and other *de novo* myelodysplastic syndrome. Partial or total loss of chromosome 5 is a frequent event in therapy-related myelodysplastic syndrome. NPM may maintain genetic stability and therefore exert tumor suppressive function.

Anton Berns induced adenocarcinomas resembling NSCLC using infection with Adeno-*Cre* and switching a floxed mutant *Ki-Ras* allele. Adeno-*Cre*–mediated inactivation of *Rb* and *p53* floxed alleles yielded tumors histologically resembling metastatic SCLC. Frequent amplification of *L-Myc* was observed in these SCLC in analogy to human SCLC. Pituitary tumors, where imaging could be applied to drug development, were induced in $Rb^{flox/flox}$ mice using pituitary-specific expression of Cre recombinase. An activated luciferase gene in those tumors allows *ex vivo* imaging of chemotherapy response. Cyclin-dependent kinase inhibitors flavopiridol or roscovitine in combination with doxorubicin were tested in retinoblastoma (Rb)–deficient cells. Drug was measured using the luciferase reporter system, and synergy was observed if doxorubicin was given 16 hours after roscovitine; thus, optimal schedules of administration would likely be clinically critical.

Julian Downward uncovered cell motility requirements and transformation by *Ras* using RNA interference libraries. Inhibitors of *Ras*-induced senescence in human ovarian surface epithelial cells were sought. MINK is a MAPK kinase kinase kinase activated by Ras through the Raf/extracellular signal-regulated kinase (ERK) pathway. Although PI3K p110α and p70 S6K1 are required for Ras-induced senescence and transformation, MINK plays a role only in Ras-induced senescence by activating p38 stress-activated kinase and p21 expression. Enhanced motility of invasive lung cancer was studied by time-lapse microscopy using A549 cells transiently transfected with RNA interference (RNAi) vectors and a green fluorescent protein vector: 1,600 genes were involved, including *CUTL1*, a transcription factor inducing a transcriptional program for cells to move rapidly. Transforming growth factor β (TGFβ) transcriptional up-regulation of *CUTL-1* increased short-term motility of NIH 3T3 cells that was blocked by *CUTL-1* siRNA. In breast cancer patients, low CUTL1 expression predicted longer survival. Using RNAi revealed downstream targets of Ras, such as MINK and CUTL-1, representing novel chemotherapy targets.

Karen Vousden discussed reactivation of p53 function as a therapeutic strategy. Understanding the choice of response to p53 activation is important to achieve maximum differential between death of cancer and survival of normal cells after p53 activation. Effects of modification of HDM2/p53 interactions, phosphorylation of HDM2, deficiency of MK2, and modulation of factor PUMA on p53 functions were outlined. In PUMA, the absence of resistance to apoptosis occurs; if enhanced by doxorubicin, apoptosis increases. E2F-1 binds to the *ASPP-1* promoter, and ASPP-1 expression is required for p53-dependent apoptosis. A novel p53 target gene (*JAVA*) encodes a protein similar to the phosphatase domain of PFK-2/FBPase-2, a principal regulator of glycolysis. *JAVA* enhances the oxidative branch of the pentose phosphate pathway, conferring resistance to oxidative stress by enhancing NADPH production and reduced glutathione levels restoration. JAVA ectopic expression protects cells from reactive oxygen species (ROS) and p53-induced cell death. JAVA was overexpressed in five human tumors. Inhibiting JAVA expression, HMD2 action or E2F-1 binding to ASPP-1 promoter, or increasing PUMA or ROS may each represent useful therapeutic interventions.

William Kaelin discussed tumor suppression by VHL protein (pVHL) as a paradigm for drug development. Germ line and sporadic mutations of *VHL* occur in renal cell carcinoma. pVHL and a ubiquitin ligase complex target the α subunit of transcription factor HIF for destruction in the presence of oxygen. Under low-oxygen conditions, or in the absence of pVHL, HIF accumulates and target genes *VEGF, PDGF B*, and *TGFβ* are transcriptionally activated. Oxygen sensing through HIF proline-hydroxylation allows recognition, and destruction of HIF, by pVHL. Increases of VEGF, PDGF, and TGFα activities and links between mammalian target of rapamycin (mTOR) and HIF suggest therapeutics against *VHL*-null tumors which include antivascular agents, PDGFR and epidermal growth factor (EGF) receptor inhibitors, and inhibitors of mTOR. A murine line was engineered to express HIF-luciferase fusion protein, allowing HIF to be imaged *in vivo* and enabling studies of molecules affecting hypoxia and HIF stability.

Philip Beachy discussed neoplastic growth promotion following dysregulated activation of hedgehog signaling. Somatic mutations

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in this pathway occur in basal cell carcinoma and medulloblastoma; recent studies with the antagonist cyclopamine revealed requirement for pathway activity in growth of SCLC and carcinomas of the esophagus, stomach, pancreas, biliary tract, prostate, and bladder. In prostate tumor, xenograft low and high hedgehog corresponded to low and high metastatic potential, respectively; hedgehog pathway manipulation induced phenotypic switching and altered invasion in Boyden chambers. Nextrin expression associated with stem cells in prostate and other hedgehog-dependent tumors was inhibited by cyclopamine. Stem cells renewal and maintenance are roles for hedgehog and Wnt pathway. Stem cell progenitor pools expand during tissue injury; chronic injury increases cancers risk through hedgehog and Wnt activity. Cancer growth resembles an active injury repair, and continuous hedgehog activity in cancers may result from stem cells' failure to return quiescent following regeneration.

Benjamin Neel outlined the role of SH2 domain-containing protein tyrosine phosphatase Shp2 and Gab2 in cancer. Shp2 germ line autosomal-dominant mutations cause Noonan syndrome. A Noonan syndrome patient subset develops myeloproliferative disorders, particularly, juvenile myelomyelogenous leukemia (JMML). Recombinant Shp2 mutants were characterized and murine models for Noonan syndrome and JMML generated. Leukemogenic transformation associated with *Shp2* mutants required Gab2 recruitment and resulted from Ras/ERK, PI3K/Akt/Tor, and signal transducers and activators of transcription 5 pathway hyperactivation. *Gab2* is overexpressed in 25% to 30% of human breast tumors. *Gab2* collaborates with antiapoptotic genes causing luminal filling reminiscent of ductal carcinoma *in situ* and with *HER-2/neu* to confer loss of acinar polarity and invasive phenotype. *Gab2* acts via Shp2 and drives hyperactivation of ERK. ERK inhibitors block Gab2-evoked proliferation and human epidermal growth factor-2 (HER2) collaboration. Gab2 levels may provide prognostic/therapeutic indices in breast cancer. Increased Shp2/Ras/ERK pathway activation by *Shp2* mutation or overexpression of Shp2 binding proteins is common in neoplasia.

Paul Workman stressed that the following was learned from recent successes with trastuzumab, imatinib, and gefitinib: (*a*) agents with potency and selectivity can be designed, but producing drugs with appropriate pharmacokinetics and pharmacodynamics is difficult; (*b*) selecting patients based on evidence of molecular dependence is critical; (*c*) developing molecular biomarkers is essential, particularly for target evaluation and drug regimens optimization; (*d*) resistance often occurs by mutation and/or overexpression of the target; (*e*) combination treatments may be essential for cancer driven by multiple oncogenic abnormalities and to prevent resistance. Multitargeted kinase inhibitors or drugs acting on single targets modulating many oncogenic genes and proteins might be useful. Inhibitors of cyclin-dependent kinases PI3K and the Hsp90 molecular chaperone were discussed. Roscovitine inhibited Rb phosphorylation, caused loss of cyclin D1, and activated the MAPK pathway. PI3K inhibitors PI 103 and LY 294002 caused G_1 arrest and apoptosis and inhibited particularly gliomas. Multiple oncoproteins are clients of Hsp90; cancer cells may depend on Hsp90 to survive stress. Geldamycin blocks ATPase activity essential for Hsp90 chaperone action; 17-(allylamino)-17-demethoxygeldanamycin (17AAG) causes cytostatic effects through Hsp90 inhibition but induces Hsp70 in melanoma. Combination of Hsp90 inhibitors with cytotoxic agents, like 17AAG plus Taxol, synergistically inhibited melanoma.

Clinical Relevance

Sylvie Menard indicated that overexpression of HER2 is associated with more aggressive breast carcinomas only in node-positive patients. Risk factors in HER2-positive cases are not associated with BRCA1 and BRCA2, irradiation, or estrogen-related risks. HER2 was overexpressed in 23% of 2,000 primary breast cancers with high numbers of mitosis. Clinical and experimental data suggested that primary tumor removal promoted metastasis growth. Growth factors were measured in wound drainage fluid and drainage of surgical damage and serum, using cultures of $HER2^+$ and $HER2^-$ cells. Trastuzumab reduced drainage fluid effects if added to cultures first. EGF is released according to circadian rhythm: the best time to perform surgery may be questioned. HER2 was associated with sensitivity to anthracyclins and resistance to hormones. Hormone receptor expression was inversely proportional to HER2. Trastuzumab is active in HER2-positive breast carcinomas and in neoadjuvant setting through antibody-dependent cell cytotoxicity, suggesting the desirability of selecting patients with appropriate immune functions.

Edith Olah analyzed breast cancer families from Hungary where five mutations were seen more than once (*BRCA1: 5382insC, C61G/300T>G*, and *185delAG; BRCA2: 9326insA* and *6174delT*). These mutations, except *6174delT*, were prevalent among 500 patients unselected for family history. Among 438 breast and/or ovarian cancer families from Hungary, Poland, Czech Republic, Serbia-Montenegro, Latvia, Greece, and Turkey, BRCA deletion was observed in 35% of females; 29 *BRCA1* and *BRCA2* sequence variants were also identified. *BRCA2* mutations were found in 33% male breast cancers. Among 12,000 cases from 699 families (8,000 unselected for family history), no differences were found between *BRCA1* and *BRCA2*.

Edison Liu indicated that computational analysis between estrogen response elements that are or are not estrogen receptor (ER) binders revealed an extended palindrome defining optimal estrogen response element. A novel technology, called gene identification signature, was developed that, when coupled with chromatin immunoprecipitation, allows for precise identification of transcription factors binding sites on a genome-wide scale. Over 1,000 binding sites and ER-responsive genes, multiple binding segments spanning many kilobases were found. A small set of validated human genes, associated with ER in breast tumors, induced ER effects in breast cancer cells. As *cis*-regulatory regions of these "core" *ER* target genes are poorly conserved, certain estrogen biological effects may differ between mouse and human more than previously thought. *ERβ* inhibits cell growth, whereas *ERα* supports it; by microarray analysis, ERα and *ERβ* were almost identical, but in *ERα*, there were differences in clustering replication genes. In the presence of *ERα*, overexpression of *ERβ* mimics hormone treatment in ligand-unexposed cells. *ERβ* expression down-regulated a small subset of genes involved in cell proliferation. The α/β ratio in target cells may have effect on receptor complex functions; ERβ is a good prognostic indicator.

Concluding Remarks

Carlo Gambacorti-Passerini identified four areas of discussion: (*a*) identification of genetic events, (*b*) assessment of gene expression patterns, (*c*) mechanisms of action of oncogenes and tumor suppressor genes, and (*d*) clinical developments. The

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different types of analyses discussed represent essential tools to clarify molecular mechanisms generating cancer. Despite the major achievements discussed, many critical questions still need to be answered.

Appendix A

The program committee consisted of the cochairs Yusuke Nakamura (Institute of Medical Science, Tokyo, Japan), Alex Matter (Novartis Institute for Tropical Diseases, Singapore), Pier Paolo Pandolfi (Memorial Sloan Kettering Cancer Center, New York, NY), and Marco Pierotti (Isituto Nazionale Tumori, Milan, Italy). In addition to the program committee members, invited participants included Philip Beachy (The Johns Hopkins University, Baltimore, MD); Anton Berns (Netherlands Cancer Institute, Amsterdam, the Netherlands); Hugues deThe (Hospital Saint-Louis, Paris, France), Julian Downward (London Research Institute, London, United Kingdom); Carlo Gambarcorti-Passerini and Sylvie Menard (Istituto Nazionale Tumori, Milan, Italy); William Kaelin, William Sellers, and Giovanni Tonon (Dana-Farber Cancer Institute, Boston, MA); Edison Liu (Genome Institute of Singapore, Singapore); Benjamin Neel (Beth Israel Deaconess Medical Center, Boston, MA); Joseph Nevins (Duke University Medical Center, Durham, NC); Edit Olah (National Institute of Oncology, Budapest, Hungary); Michael Stratton (The Wellcome Trust Sanger Institute, Cambridge, United Kingdom); Karen Vousden (Beatson Institute for Cancer Research, Glasgow, Scotland, United Kingdom); and Paul Workman (Institute of Cancer Research, Sutton, Surrey, United Kingdom).

The posters were presented by M.P. Colombo (Istituto Nazionale Tumori, Milan, Italy), P. Parrella (Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy), K. Scotlandi (Istituti Ortopedici Rizzoli, Bologna, Italy), S. Buzzi (Tris Medical Center, Ravenna, Italy), F. Polato and E. Marrazzo (Istituto di Ricerche Farmacologiche "Mario Negri," Milan, Italy), D. Demarquay (IPSEN-Institut Henri Beaufour, Les Ulis, France), M. Angela (University of Pisa and Pisa University Hospital, Pisa, Italy), O. Balacescu and I. Berindan Neagoe (Cancer Institute, Cluj-Napoca, Romania), F. Cavallo (University of Turin, Orbassano, Italy), M. Zavaglia (University of Pisa, Pisa, Italy), L. Brescacin (University of Padua, Padua, Italy), C. Potrich (Consiglio Nazionale delle Ricerche-ITC Istituto di Biofisica Sezione di Trento, Povo, Italy), and A. Sartore-Bianchi (Ospedale Niguarda Ca'Granda, Milan, Italy).

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