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Validating Somatic Mutations Discovered Through Genetic Analysis of Peripheral T-Cell Lymphomas (PTCLs).

A) Study Proposal and Rationale:

Peripheral T-cell lymphomas (PTCL) are a relatively rare subset of non-Hodgkin lymphomas, occurring with a frequency of about 0.78 per 100,000 person-years in the United States (1). PTCLs are a heterogeneous group subdivided into multiple subtypes, with the most common subtype being PTCL-NOS (through histologic exclusion of other types), followed by anaplastic large cell lymphoma (ALCL), cutaneous T-cell lymphoma (CTCL), angioimmunoblastic T-cell lymphoma (AITL), and several rarer subtypes (2). Aside from CTCL, most PTCLs are aggressive with poor response to therapy, with 10 year OS ranging from ~20% for AITL and PTCL-NOS to 50-70% for ALCL. Prognostic and treatment efforts have been complicated by the relatively low number of cases and by difficulty in accurately classifying the subtypes. The importance of trying to better understand the genetics of PTCLs is underscored by the discovery of anaplastic lymphoma kinase (ALK) in ALCL. ALK+ ALCL has a significantly better outcome than ALK- tumors, and ALK has been the target of multiple targeted therapies (3).

Because PTCLs are poorly understood at a molecular level, significant efforts are under way to utilize whole exome and whole genome sequencing and RNA expression studies to provide a more accurate way to classify and diagnose the subsets, to provide better prognostic tools, and to look for novel therapeutic targets. A recent study evaluating mRNA expression signatures for 372 PTCL cases showed that classification using gene expression was feasible and more accurate than histologic diagnosis, and revealed several prognostic gene signatures (4). Studies by the Ferrando lab and others have also focused on sequencing of tumor-normal pairs of PTCL cases. Palomero et al used a combination of whole-exome sequencing, RNA sequencing, and targeted deep sequencing to characterize somatic mutations in 12 tumor-normal tissue pairs, followed by validation in more tumor samples (5). Whole-exome sequencing showed a total of 288 mutations in 268 genes, averaging 24 somatic mutations per tumor. Mutations occurred in epigenetic regulators previously implicated in oncogenesis, including TET2, TET3, DNMT3A, IDH2, SETD2, as well as other putative oncogenes and tumor suppressors such as ATM, DNAH5, TACC2, RYR3, PTPRD and MGAT4C. Mutations were also found in proteins that mediate T-cell signaling, such as FYN, and immune-related genes such as B2M and CD58. Finally, sequencing found a novel dominant negative mutation G17V in the small GTPase RHOA that was highly prevalent in AITL and PTCL-NOS. Similar studies from other groups also found these mutations (6). These studies show the utility of genetic analysis in characterizing PTCLs and discovering new candidate mutations for further experiments. The goal of the proposed study is to further characterize somatic mutations in PTCLs found by Dr. Ferrando's lab.

Hypothesis:

The hypothesis is that mutations found in multiple tumor samples (but not in matched normal tissue) contribute to the pathogenesis of PTCLs via effects on cell growth and resistance to apoptosis. Expression of mutants that function as constitutively active or dominant negative proteins will alter these parameters in T-cell lymphoma cell lines.

B) Study Design and Statistical Analysis:

Selection of candidate mutations:

We will first generate a priority list of mutations to analyze by ranking mutations in order of frequency in the data set generated in Palomero et al (5), as well as in currently ongoing sequencing efforts in the lab. We will use 1) novel mutations in proteins previously shown to function as tumor suppressors or oncogenes, 2) high-frequency mutations in proteins not previously known to be involved in oncogenesis, and 3) control mutants already validated from prior PTCL sequencing experiments, such as the RHOA G17V mutation that has been shown to alter proliferation relative to wild type in Jurkat cells (6).

Retroviral expression of mutant and wild-type candidate proteins:

The pMSCV-PIG (puro-IRES-GFP) retroviral vector has been previously used in multiple hematopoietic lineages including T-cell lines such as Jurkat, and allows for selection of cells with puromycin as well as tracking using GFP fluorescence. Wild-type candidate protein cDNAs will be cloned into this vector and targeted mutagenesis will be used to generate candidate mutations.

T-lymphoma cell lines:

Jurkat cells are an immortalized human T-cell leukemia line that can recapitulate leukemia when injected intravenously or lymphoma when injected subcutaneously in immunosuppressed mice. These will be the primary cells used for experiments. In addition, to test mutations on different tumor genetic backgrounds, we will use CUTLL1 cells, a T-cell lymphoma line established by the Ferrando lab (7), and the CTCL cell lines HuT78 and HuT102.

Cell culture experiments:

Jurkat cells will be transduced with retrovirus expressing either wild-type cDNAs as control or with mutant cDNAs. Infected cells will be selected using puromycin. We will assess cell growth by serial counting of viable cells in culture. To assess proliferation, we will do cell cycle analysis by incorporation of BRDU and DNA staining with 7-AAD, and quantify the percentage of cells in G1, S, and G2 phases by flow cytometry. To assess apoptosis, we will quantify cells that externalize phosphatidylserine using fluorescently labeled AnnexinV, and quantify annexin positive apoptotic cells using flow cytometry. All experiments will be done in triplicate, and cell numbers, % of cells in S-phase, and % apoptotic cells will be determined for each sample as a continuous variable.

Mouse xenograft experiments:

We will test the mutations with most significant effects in cell culture experiments using two mouse xenograft models. Jurkat cells will be infected with control or test retrovirus as above. For intravenous injection experiments, we will use irradiated NOD/scid/gamma (NSG mice). We will inject 5 million cells per mouse for a total of 10 mice per group (8). Mice will be monitored daily and sacrificed when exhibiting clinical signs of leukemia. Mean survival will be determined for each group. For flank tumor model, we will inject 50 million cells into one flank of irradiated nude mice, for a total of 10 mice per group (9). Tumor size will be measured daily and mice will be sacrificed at 14 days or when exhibiting signs of clinical illness. Mean tumor size at each day of measurement will be determined for each group.

Statistical analysis:

The null hypothesis for each experiment is that there is no difference between expression of wild-type and mutant candidate protein. For cell culture experiments, data will be expressed as average and standard deviation of three experiments. For mouse experiments, data will be expressed as average and standard deviation of the mice in each group. For experiments involving a single mutant versus wild-type control, two-tailed unpaired t-test will be used to determine p-value (with threshold for statistical significance of $p < 0.05$. For experiments comparing multiple mutants to each other and to control wild-type protein, ANOVA will be used. For *in vivo* experiments with intravenous injection of cells, using 10 mice per group, with an estimated mean survival of 30 days for controls and SD of 10 based on prior studies, we can detect mean survival difference of 15 days (50% change) with α of 0.05 and power of 0.90. For *in vivo* experiments with subcutaneous injection of cells, using 10 mice per group, with an estimated mean tumor size of 150 mm^3 at 14 days for controls and SD of 25 based on prior studies, we can detect mean tumor size difference of 40 mm^3 (25-30% change) with α of 0.05 and power of 0.90.

References:

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