Xiaoxian Zhao, PhD, (l) and Eric Hsi, MD, confirmed the potential of CLL-1 as a target for antibody-based therapeutics in the treatment of AML using preclinical in vitro models.

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Feature Story

Targeting C-Type Lectin-Like Molecule-1 in Acute Myeloid Leukemia

By Xiaoxian Zhao, PhD, and Eric D. Hsi, MD

Background

Acute myeloid leukemia (AML) is the most common acute leukemia affecting adults. While modern chemotherapy regimens can induce complete remission in the majority of patients, the overall prognosis is still poor with median survival of less than two years (1). Therapies using monoclonal antibodies (mAbs) as a targeted approach to eliminate leukemic cells have proven to be a useful strategy. Gemtuzumab ozogamicin is a toxin-conjugated mAb that binds to CD33 on myeloid leukemia blasts and is approved for the treatment of patients over 60 years old with relapsed AML. The overall response rate for gemtuzumab ozogamicin is approximately 30 percent in clinical studies (2, 3). Due to the success of this and other immunotherapies with humanized mAbs, investigators are searching for additional targets.

C-type lectin-like molecule-1 (CLL-1) is a transmembrane receptor expressed on myeloid cells and AML blasts (4). In collaboration with investigators at Nuvelo, we used a series of novel mAbs against the extracellular domain of CLL-1, provided detailed expression analysis of CLL-1 in normal and AML samples and assessed cytotoxicity of selected human/murine chimeric anti-CLL-1 mAbs in preclinical in vitro models (5).

Validation of CLL-1 as a Potential Therapeutic Target

Flow cytometric analysis showed CLL-1 expression in 45 of 52 AML cases (86.5%). The mean percentage of blasts expressing CLL-1 was 70% (range 23-99%). Differences in frequency of expression occurred based on AML type with the highest incidence (100%) in cases with myelomonocytic and monocytic maturation (Table 1). Immunohistochemical CLL-1 staining of AML bone marrow by tissue microarray showed expression in 37 out of 38 cases (Fig. 1). In contrast, CLL-1 expression was not detected in blasts from acute lymphocytic leukemia (ALL, n=5).

Next, we analyzed CLL-1 expression in the CD34+/CD38- compartment, enriched for leukemic stem cells. CLL-1 was detected in this compartment in 12/22 of these AML cases (54.5%). CD34+ progenitor cells from samples lacking hematologic malignancy (n=8) showed two cases expressed CLL-1 (23% and 59% positive cells). CD34+/CD38- bone marrow-derived stem cells from five such samples showed that only one case expressed CLL-1 in a subset (21%) of cells.

<table>
<thead>
<tr>
<th>Table 1. CLL-1 expression in blasts of AML, MDS and ALL</th>
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<tr>
<td><strong>AML subtype</strong></td>
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<tr>
<td>---------------------------------------------------------</td>
</tr>
<tr>
<td>AML with CBFβ/MHb/1</td>
</tr>
<tr>
<td>AML with PML/RARα</td>
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<tr>
<td>AML with 11q23 abnormality</td>
</tr>
<tr>
<td>AML with multilineage dysplasia</td>
</tr>
<tr>
<td>AML minimally differentiated</td>
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<tr>
<td>AML without maturation</td>
</tr>
<tr>
<td>AML with maturation</td>
</tr>
<tr>
<td>Acute myelomonocytic leukemia</td>
</tr>
<tr>
<td>Acute monoblastic and monocytic leukemia</td>
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<tr>
<td>MDS</td>
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<tr>
<td>ALL</td>
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Additionally, we surveyed CLL-1 expression in either bone marrow or peripheral blood blasts from 13 cases of myelodysplastic syndrome (MDS). A total of six cases were positive (46.2%) with a mean of 50% positive cells (range 21-85%, Table 1). Among the 13 MDS cases, one refractory anemia and all five RAEB-1 cases were negative for CLL-1 expression. Of three cases of RAEB-2, two cases were positive; one MDS/MPD, one therapy-related MDS, one MDS 5q(-) syndrome, and one MDS unclassifiable case were all positive.

We next compared the expression pattern of CLL-1 with the myeloid antigen CD33. Both were highly expressed in monocytes with lower expression in granulocytes. Lymphocytes from AML or normal controls did not express CLL-1.

These studies confirm the restricted expression of CLL-1 in cells of myeloid lineage, in the majority of AML blasts, and in a subset of CD34+/CD38- leukemia cells. This supports the potential of CLL-1 as a target for antibody-based therapeutics in the treatment of AML.

**Functional Assay of Anti-CLL-1 mAb**

To access the therapeutic potential of anti-CLL-1 mAbs, we performed cytotoxic activity assay via complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). Lead chimeric mAbs against CLL-1 displayed a dose-dependent CDC activity against CLL-1+ AML-derived cell line OCI-AML5 and eliminated more than 80% of target cells at doses of 100 ng/ml and higher. These same mAb had no activity against the Burkitt lymphoma B cell line CA46, which is negative for CLL-1.

Furthermore, CDC assays on AML blasts showed a dose-dependent activity against 15 of 16 (94%) CLL-1 positive samples. The percentage of lysed cells ranged from 25 to 85% (Fig. 2A). In contrast, ALL blasts lacking CLL-1 were insensitive to CDC-mediated activity.

![Figure 1. Normal and AML bone marrow stained with CLL-1 mAb.](image)

![Figure 2. CDC activity of chimeric mAb against fresh isolate AML blasts (A) and ADCC assay of anti-CLL-1 chimeric mAb against HL-60 cells (B)](image)

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Spotlight on Staff

Carol Farver, MD, Department of Anatomic Pathology and Pathology & Laboratory Medicine Institute Vice-Chair for Education, became the first recipient of the Distinguished Achievement Award for Graduate Medical Education given by the Association of Pathology Chairs at their annual meeting in Seattle, Wash. in July. Dr. Farver was selected by the Association’s Graduate Medical Education Committee and the Program Directors Section selected Dr. Farver as the honoree for her outstanding contributions to graduate medical education.

Gary W. Procop, MD, MS, Chair, Department of Molecular Pathology, presented “The Use of High Resolution Melt Curve Analysis, Pyrosequencing, and DNA PROFiling to Identify and Characterize Microorganisms” and moderated a session on “Emerging and Novel Technologies for Infectious Molecular Diagnostics” at Cambridge Healthtech Institute’s Next Generation Dx Summit held in Washington, DC August 24-26. The Summit brought together all of the major players in the molecular diagnostics arena to showcase advances in technology, research and diagnostic development, with an emphasis on applications to meet clinical demands.

Kandice Kotke-Marchant, MD, PhD, was co-director for the College of American Pathologists (CAP) Annual Meeting held in Chicago September 24-29 with John Olson, MD, PhD, Professor, Department of Pathology, University of Texas, San Antonio. Dr. Marchant and Dr. Olson presented a half-day course, “An Algorithmic Approach to Hemostasis Testing,” based on their CAP textbook An Algorithmic Approach to Hemostasis Diagnosis.

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ADCC induced by humanized mAbs is an important in vivo mechanism of action for therapeutic antibodies such as rituximab. To evaluate the ability of anti-CLL-1 chimeric antibody to elicit ADCC activity, HL-60 cells were labeled and incubated with freshly isolated human NK cells in the presence of chimeric mAb. A dose-dependent ADCC-mediated lysis was observed when using chimeric mAb 1075.7, whereas an isotype control mAb had no detectable activity (Fig. 2B). Nearly 30% of cells were lysed with an EC50 of 20 ng/ml.

Summary
Selective expression of cell surface antigens on target cells provides opportunity for antibody-based immunotherapy both for leukemia and solid tumors. CLL-1 is a novel receptor expressed on the majority of leukemic blast cells and in populations enriched for leukemia stem cells. Selected unconjugated (naked) chimeric anti-CLL-1 mAbs displayed potent and selective CDC and ADCC in CLL-1 expressing cells. CLL-1 is a promising target for therapeutic mAbs.

References
The BRAF protein plays a central role in cancer cell growth and survival. BRAF is a downstream effector of the RAS/RAF/MAPK/MEK signaling pathway. Signal transduction of this pathway often is initiated via ligand binding of transmembrane receptors such as epidermal growth factor receptor (EGFR) or platelet-derived growth factor receptor (PDGFR), but many upstream activators of the RAS/RAF/MAPK/MEK pathway exist.

The T1799A point mutation causes the V600E transversion in exon 15 of the \textit{BRAF} gene located at the 7q34 locus, a valine-to-glutamine mutation that accounts for the vast majority of oncogenic \textit{BRAF} mutations. \textit{BRAF} mutational analysis serves several different purposes in clinical practice.

\textbf{Lynch Syndrome/HNPCC Screening}

Lynch syndrome (LS)/hereditary non-polyposis carcinoma (HNPCC) is an autosomal dominant cancer syndrome and represents the most common cause of inherited colorectal cancer, accounting for approximately 2 to 5 percent of newly diagnosed colorectal cancers. Patients with this syndrome have an up to 80 percent lifetime incidence of colorectal cancer and an increased risk for endometrial, skin, urinary tract, ovarian, small intestinal, biliary, and gastric cancers, among others. High microsatellite instability (MSI-H) characterizes about 95 percent of colorectal carcinomas occurring in the setting of LS/HNPCC and serves as a surrogate marker of DNA mismatch repair deficiency. The DNA mismatch repair machinery is encoded by a number of genes, with \textit{MLH1}, \textit{MSH2}, \textit{MSH6}, and \textit{PMS2}, accounting for about 95 percent of the identifiable causative mutations in LS/HNPCC. The Bethesda guidelines (Table 1) outline screening recommendations for detecting LS/HNPCC patients via MSI testing or immunohistochemistry.

Unfortunately, the Bethesda guidelines may miss up to 25 percent of LS/HNPCC patients, usually in older patients and in those with a limited family history. Due to this imperfect sensitivity, Cleveland Clinic has committed to universal screening for LS/HNPCC. MSI testing on pre-operative samples is preferred as an initial screen because it is the most sensitive and may help guide the extent of surgical resection. An MSI-H phenotype is not diagnostic of LS/HNPCC because some 10 to 15 percent of sporadic colorectal carcinomas also demonstrate MSI-H, almost always due to epigenetic silencing of the \textit{MLH1} promoter. Likewise, both sporadic and HNPCC-associated MSI-H colorectal carcinomas can demonstrate loss of \textit{MLH1} expression by immunohistochemistry; however, loss of expression of \textit{MSH2}, \textit{MSH6}, or isolated \textit{PMS2} points much more strongly to LS/HNPCC than to sporadic cancers.

\begin{table}[h]
\centering
\begin{tabular}{|l|}
\hline
\textbf{Colorectal carcinoma (CRC) diagnosed in a patient < 50 years.} \\
\hline
\textbf{Synchronous or metachronous CRC or other HNPCC-associated tumors (colorectal, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract, brain, skin, and small bowel), regardless of age.} \\
\hline
\textbf{CRC with MSI-H histology (tumor infiltrating lymphocytes, Crohn’s-like reaction, mucinous differentiation, or medullary growth pattern) in patient < 60 years.} \\
\hline
\textbf{CRC diagnosed in a patient with at least 1 first-degree relative with an HNPCC-associated tumor, one tumor diagnosed at < 50 years.} \\
\hline
\textbf{CRC diagnosed in a patient with at least 2 first-degree or second-degree relatives with HNPCC-associated tumors, regardless of age.} \\
\hline
\end{tabular}
\caption{Revised Bethesda Guidelines: Any criterion warrants clinical testing.}
\end{table}
Figure 1. H&E-stained section from a 54-year-old female with colon cancer. The colonic carcinoma shows several histologic features associated with MSI-H, including mucinous differentiation and tumor infiltrating lymphocytes.

Figure 2. MLH1 immunohistochemistry shows loss of expression in the carcinoma nuclei. This may be a sporadic or LS/HNPCC-associated carcinoma. The background stromal and inflammatory cells retain normal nuclear expression.

Figure 3. BRAF mutation testing shows two peaks at codon 600 (arrow), representing a heterozygous allele. One peak represents the normal allele and the other the TÀA transversion that characterizes the V600E mutation. This is strong evidence that the patient’s tumor is sporadic rather than LS/HNPCC-associated.

Gene sequencing all carcinomas with MLH1 loss would be unnecessarily laborious and expensive. In addition, only about one in 10 MLH1-loss patients are secondary to LS/HNPCC, and BRAF mutation testing can help exclude at least half of these patients. BRAF V600E mutations occur in about 40 to 50 percent of sporadic MSI-H colorectal carcinomas, but until recently, these mutations were never reported in HNPCC-associated carcinomas. PMS2-mutated carcinomas with concomitant BRAF V600E mutations have been reported recently, which can be differentiated from sporadic MSI-H carcinomas by virtue of intact MLH1 immunohistochemical expression.

Prognostic/Predictive Marker in Metastatic Colorectal Carcinoma

Several retrospective studies have found that tumors harboring BRAF V600E mutations do not respond to anti-EGFR monoclonal antibody therapy, similar to KRAS codon 12/13 mutations. Anti-EGFR therapies are extremely expensive, have side effects, and may delay the use of other, more effective treatments in these patients. In these retrospective analyses, 38 patients with metastatic colorectal cancer harboring BRAF V600E mutations were treated with anti-EGFR therapy, and none of the patients demonstrated an objective response (0/38).
This predictive role of **BRAF** mutation has come under question recently, however, after an abstract presented at the American Society of Clinical Oncology Gastrointestinal Cancers Symposium in January 2010 using prospective data. Accordingly, the National Comprehensive Cancer Network (NCCN) views **BRAF** mutation testing in metastatic colorectal cancer as optional, and this test is not routinely performed on colorectal cancers at Cleveland Clinic.

**BRAF** V600E also has prognostic value in colorectal cancer. Patients with metastatic colorectal carcinoma or microsatellite stable (MSS), non-metastatic colorectal carcinoma harboring **BRAF** V600E mutations have been found to have significantly worse overall survival, progression-free survival, and response rates to conventional chemotherapy.

**Other Cancer Types**

**BRAF** is the most commonly mutated gene in papillary thyroid carcinoma (PTC), occurring in approximately 45 percent of tumors. Greater than 95 percent of **BRAF** mutations in PTC are the V600E transversion. **BRAF** mutations usually are encountered in PTC with conventional or tall cell histology, whereas **BRAF** mutation in the follicular variant of PTC is uncommon. **BRAF** mutations are not seen in follicular neoplasms, making **BRAF** mutations a good marker of PTC. Additionally, **BRAF** V600E mutations have been correlated with aggressive histologic features in PTC, poor treatment outcomes, tumor recurrence, and tumor-related death. At some centers, **BRAF** mutational analysis is performed on preoperative biopsy samples to help confirm the diagnosis of PTC, triage non-diagnostic samples, and assist the surgeon in determining the extent of surgical resection and/or lymph node dissection.

**BRAF** mutations occur in 40 to 70 percent of cutaneous melanomas, with V600E mutations accounting for more than 90 percent of mutations. **BRAF** mutations seem to predict clinical response to either **BRAF** or MEK inhibitors in melanoma and other tumors, and numerous clinical trials of **BRAF** (e.g. PLX4032) and MEK (e.g. AZD6244) inhibitors in melanoma, thyroid, and other solid tumors are under way. These trials frequently analyze **BRAF** mutation status or are restricted to **BRAF**-mutated tumors, so pre-trial mutational analysis often is necessary.

**Limitations of the Assay**

In practice, the lower limit of reliable mutation detection is 25 percent tumor cells. Mutations may not be detected in samples with abundant dilution by non-tumor DNA. This is particularly relevant in the post-adjuvant therapy setting.

**Suggested Readings**


About the Authors

**Eric Hsi, MD**, is the Chair of the Department of Clinical Pathology in the Institute of Pathology & Laboratory Medicine. A graduate of the University of Michigan School of Medicine, he completed a residency in pathology and a fellowship in surgical pathology at the University of Michigan Health System hospitals. Dr. Hsi is board-certified in anatomic pathology, clinical pathology and hematopathology. His specialty interests include leukemia and lymphoma, immunophenotyping, molecular diagnostics and development of new therapeutics.

**Thomas Plesec, MD**, is an associate staff member in the Department of Anatomic Pathology in the Institute of Pathology & Laboratory Medicine. He earned his medical degree from Northeastern Ohio Universities College of Medicine followed by a residency in anatomic and clinical pathology and a fellowship in GI pathology at Cleveland Clinic. He is board-certified in anatomic and clinical pathology, and his specialty interests include GI and hepatic pathology.

**Xiaoxian Zhao, PhD**, is a member of the project staff in the Department of Clinical Pathology in the Institute of Pathology & Laboratory Medicine. His specialty interest is in molecular diagnostics and new biomarkers in hematopathology. Dr. Zhao completed his undergraduate degree at Shandong Medical University in Jinan, China and a doctorate and research fellowship at the University of Tsukuba, Japan.