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Cholangiocarcinoma is an uncommon but highly aggressive malignancy that arises intra- or extra-hepatically from the biliary ductal epithelium.

There is evidence that the neoplastic transformation of biliary epithelial cells and malignant progression of cholangiocarcinoma is accompanied by a number of genetic and epigenetic alterations (1, 2). Genetic alterations such as point mutations of K-ras and p53 have been found in a subset of cholangiocarcinoma (3), and mutation or deletion of p14ARF and p16INK4a were not frequent events in cholangiocarcinoma (4). Although over-expression of B-catenin was frequently encountered in cholangiocarcinoma, mutation of B-catenin has not been identified to date (5). These studies indicate that genetic alterations frequently seen in other epithelial cancers are not commonly seen in cholangiocarcinoma.

Aberrant methylation of promoter CpG islands associated with human tumor suppressor genes has been studied as an alternative mechanism of gene inactivation that contributes to the biology of several human neoplasms (6,7). Recent studies suggest that both epigenetic and genetic alterations are involved in carcinogenesis of biliary epithelium (8-11).

To advance the understanding of the epigenetic alterations in cholangiocarcinoma, we have studied the methylation profiles of 12 candidate tumor suppressor genes, APC, CDH-1/E-cadherin, O6-methylguanine methyltransferase (MGMT), ras-associated secreted factor (RASSF1A), glutathione S-transferase (GSTP), retinoic acid receptor beta-2 (RAR-B), p14ARF, p15INK4B, p16INK4A, p73, hMLH-1, and death-associated protein kinase (DAPK), in 72 cases of cholangiocarcinoma compared to 10 cases of benign biliary epithelia.

Methylation of Tumor Suppressor Genes

Our study demonstrates that promoter hypermethylation of tumor suppressor genes is a frequent event in cholangiocarcinoma. About 85 percent of cholangiocarcinoma had methylation of at least one of the tumor suppressor genes that we studied. Although methylation
of each of 12 tumor suppressor gene promoters was seen in cholangiocarcinoma, the most frequently methylated tumor suppressor genes were RASSF1A (65.3%), p15INK4b (50%), p16INK4a (50%), APC (45.8%), and E-cadherin (43%).

One of the important findings from our study is that cholangiocarcinoma often had concurrent methylation of multiple tumor suppressor genes.

With the exception of one case, methylation of two or more tumor suppressor gene promoters was present in all methylated cases. Approximately two-thirds of cholangiocarcinoma harbored methylation of three or more tumor suppressor gene promoters, and more than half of the cases had methylation of four or more tumor suppressor gene promoters.

It appears that carcinogenesis of cholangiocarcinoma may involve the disruption of multiple signaling transduction pathways, such as the ras pathway (RASSF1A), Wnt pathway (APC and E-cadherin), RB pathway (p16INK4a), cell cycle control (p15INK4b), p53 pathway (p14ARF and p73), and microsatellite stability (hMLH-1). Our data further indicate that accumulation of such epigenetic alterations through concerted methylation of multiple tumor suppressor genes is required during carcinogenesis of the biliary epithelium (12).

Enhancing Diagnostic Accuracy

Accurate diagnosis of cholangiocarcinoma in cytologic specimens, such as biliary brushing and fine needle aspiration biopsy, is notoriously challenging for pathologists. One of the main reasons is that most cholangiocarcinomas are well-differentiated histopathologically and have some morphologic features that overlap with reactive biliary epithelium. As concurrent methylation of multiple tumor suppressor gene promoters was not seen in benign biliary epithelium, a multiple tumor suppressor gene methylation pattern in cholangiocarcinoma appears to be useful in facilitating the diagnosis of cholangiocarcinoma in preoperative biopsy or biliary brushing samples. To test the plausibility of utilizing methylation profiling in detection of cholangiocarcinoma in cytologic specimens, we have retrospectively collected 35 biliary brushings and analyzed methylation profiles of the 14 tumor suppressor genes mentioned above using methylation-specific polymerase chain reaction (PCR).

Based on subsequent follow-up through surgical biopsy, surgical resection, or subsequent cytologic results, 35 biliary brushings include 27 reactive biliary epithelia and 8 cholangiocarcinomas. The initial cytologic diagnosis is summarized in Table 1. Briefly, the initial cytologic diagnoses include 15 negative, five positive, and 15 atypical. Fifteen cytologically atypical cases include 80% (12/15) of reactive biliary epithelia and 20% (3/15) of malignant biliary epithelia. Among 27 reactive biliary epithelia, 56% (15/27) were correctly diagnosed as negative by cytology (specificity), 44% (12/27) were diagnosed as atypical, and none were diagnosed as positive. In the cases of cholangiocarcinoma, 63% (5/8) of cancer cases were correctly diagnosed by cytology alone (sensitivity), and 37% (3/8) were diagnosed as atypical not otherwise specified. Overall sensitivity and specificity in detecting cholangiocarcinoma by cytomorphology alone were 63% and 56%, respectively. Approximately 38% of cholangiocarcinoma and 44% of reactive biliary epithelia yield uncertain results due to the overlapping cytomorphologic features between benign/reactive and well-differentiated malignant biliary epithelial cells.

Study of methylation profiles showed that frequent methylation of tumor suppressor genes was seen in all eight cases of cholangiocarcinoma in comparison to reactive cases. Inconsistent with our previous findings in surgical specimens, methylation of three or more tumor suppressor genes was seen in all eight cases of cholangiocarcinoma. Although scattered methylation of single and...

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occasional double tumor suppressor genes was seen in some of the cases with reactive biliary epithelia, methylation of more than three tumor suppressor genes was not seen in any of the 27 cases of reactive biliary epithelia.

Using methylation profiling of multiple (greater than three) tumor suppressor genes, we correctly predicted all eight cases of cholangiocarcinoma, including those three cytologically “atypical” cases by cytology. Our study further confirms that promoter methylation of multiple tumor suppressor genes is the hallmark of malignant biliary epithelium, and methylation profiling can be a useful molecular tool in facilitating accurate diagnosis of cholangiocarcinoma in biliary brushings.

To assess the utility of methylation profiling in diagnostic pathology, it is important to study the background benign tissue surrounding lesions. We have studied the plausibility of clinical application using a methylation-specific PCR technique in the differential diagnosis of cholangiocarcinoma vs. reactive atypia in cytologic samples. Our data show that although single or double gene methylation profiles can be seen in benign biliary epithelium, it is the concurrent multiple gene methylation profiling that may be useful clinically in distinguishing malignant from benign biliary epithelia.

References

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Luminex xMAP

Versatile Bioassay Technology Simultaneously Detects and Quantitates Multiple Analytes in a Single Sample

By Belinda Yen-Lieberman, Ph.D.

Luminex xMAP (Luminex, Austin, Tex.) technology is a homogenous, multiplexed system that is capable of making simultaneous analysis of up to 100 analytes on a single specimen.

This method allows internal standard curves and tests for several different analytes, such as rheumatoid factors, heterophil antibodies, and enzymes to be measured on every patient sample. It has proven to be cost-effective and eliminates "quantity not sufficient " (QNS)-qualifier on patient samples in the clinical laboratory.

The Luminex's xMAP technology uses polystyrene microspheres that contain two spectrally distinct dyes. By using precise ratios of the fluorochromes contained in each bead, an array of 100 distinct beads can be detected, with each bead-set signifying an assay. When a third fluorochrome is coupled to the bead surface via reactant binding, it becomes possible to detect binding to as many as 100 different targets in one well. Assays can include enzymatic assays, reporter-legand binding assays, transcriptional-profiling, and immunoassays.

The Luminex HTS (High Throughput System) instrument combines the sample throughput with a high-content screening system to produce an assay platform capable of generating more than 400,000 data points per day. By incorporating xMAP assay technology, fluidics, and integration with a standard eight-probe liquid handler, the Luminex HTS can simultaneously analyze 48 samples for one analyte, one sample for 50 analytes, or any combination in between.

The AtheNA Multi-Lyte MMRV Test system (Inverness Medical Innovations, Waltham, Mass.) is a multiplexed, homogenous, fluorescent-based microparticle immunoassay designed to detect, quantify, and distinguish the presence of measles, mumps, rubella, and varicella-zoster viruses (MMRV) antibodies from a single serum sample using the xMAP technology and the Luminex 100 IS Instrument. Combining the software with real-time data analysis, we were able to compare the serology profiles on measles, mumps, rubella, and varicella-zoster viruses of 144 clinical samples with the in-house VIDAS system for test concordance, ease of use, hands-on time, and turnaround time.

The Multi-Lyte System (xMAP) uses a multiplexed, fluorescent, bead-based method that is designed to simultaneously perform multiple antibody-detection in a single well with a single sample. These color-coded bead sets have been conjugated with specific viral antigens to be tested. Thousands of bead sets are combined to form a multiplex bead suspension. The reacted-bead suspension is analyzed using the Luminex 100, which uses the flow-cell and light-scatter technology for resulting.

Results of our study showed high agreement (MMRV at 100%, 100%, 100%, and 97.9%, respectively) with our in-house VIDAS test system. Luminex xMAP technology offers a significant reduction in hands-on time and turnaround-time, significant cost savings, and elimination of QNS-qualifiers in a busy clinical laboratory.

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In addition to the multiplexed immunoassays, Luminex technology also has demonstrated several rapid, high-throughput methods for single nucleotide polymorphism (SNP) assay for tissue typing. Most recently, the Luminex xMAP technology was used for the development of a rapid multiplex test for the identification of 17 high-risk human papillomavirus (HPV) types from high-risk HPV positive (HC2 assay) cervical specimens. Studies have shown that high-risk HPVs are more often associated with cervical cancers in women. Rapid methods to genotype HPV would be beneficial to guide the implementation of routine screening assays and vaccines. This rapid multiplexed genotyping assay for high-risk HPV genomes was developed based on general primer PCR-amplified fragments of the L1 region of HPV. Cloned HPV genomes were used as the template for the analytical assessments. A 142 bp region of the L1 was amplified using a modified version of the GP5+/6+ primers, and amplicons were detected using a liquid-based bead array following hybridization to type-specific oligonucleotide probes. The capture probes for HPV types 06, 11, 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73, and 82 were formulated in the cocktails, allowing the specific identification of all 19 genotypes in a single hybridization reaction. The multiplex HPV genotyping of up to 80 samples can be completed in a single day.

The multiplex HPV genotype assay using Luminex xMAP technology could easily be used in the clinical laboratory to identify type-specific, persistent infections and better predict high-grade disease or cancer following a positive HPV screening test.

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Hepatocellular Carcinoma
Genomic Alterations in Random Biopsies of Hepatitis C Cirrhosis Identify HCC Patients

By Lisa Yerian, M.D.

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide, with more than 500,000 cases diagnosed each year (1). Patients with cirrhosis related to chronic hepatitis C virus infection (HCV) are at increased risk for hepatocellular carcinoma (HCC), but no adequate screening tests yet exist to identify the approximately 10 percent minority who develop HCC. This is an important clinical issue because most HCC patients present with unresectable disease; for the few who are eligible for surgical resection, recurrence rates can be as high as 50 percent at two years and survival as brief as 12 months (2). Current screening protocols for HCC include serum AFP testing and ultrasound, but these tests have inadequate sensitivity and specificity in patients with chronic hepatitis and cirrhosis (3). Better biomarkers of HCC clearly are needed.

The molecular biology of HCC is quite complex, involving alterations of virtually every major tumorigenic pathway. The most frequently reported molecular alterations in HCC include p53 mutation, telomere shortening, and genomic instability characterized by chromosomal gains and losses. We hypothesized that the diffuse inflammatory damage imparted by HCV induces similarly organ-wide genomic injury that could serve as a biomarker to differentiate patients with HCC (“progressors”) from those without it (“nonprogressors”), and that such biomarkers may ultimately predict occurrence of HCC prior to clinical diagnosis. To identify potential biomarkers of HCC in HCV cirrhosis, we have studied benign nontumorous cirrhotic liver from patients undergoing liver transplantation for HCV cirrhosis. Our studies to date have included 39 patients: 23 progressors with HCC and 16 nonprogressors.

p53
The most frequently altered gene in human tumors, the p53 tumor suppressor gene is reported to be altered in approximately 30 percent of HCC patients (3). We analyzed p53 status in hepatocyte preparations from random nontumorous samples of cirrhotic liver, HCC, and normal control livers using chromosome 17 centromere and p53 arm DNA fluorescence in situ hybridization (FISH) probes.

FISH analysis identified non-clonal alterations, as differing abnormalities were documented in small numbers of individual cells that in composite are informative. Loss of p53 was identified in a mean of 3.8% of cells from nonprogressors versus a mean of 8.0% of cells from progressors (two-sided Wilcoxon rank sum test P<0.005). Gains of p53 and centromere abnormalities did not differ significantly between progressors and nonprogressors. Normal liver controls demonstrated p53 loss in a mean of 1.5% of cells.

Telomere Length
Telomere shortening and telomerase activation have been widely reported in HCC and other human malignancies. We evaluated telomere length in samples of benign cirrhotic liver by quantitative polymerase chain reaction (PCR), generating a ratio between the quantity of telomeric DNA and the quantity of a single copy control gene (4).

Average telomere length values for nonprogressor cirrhotic livers

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were 0.922 versus 1.183 in progressors (Satterthwaite t-test P<0.001). Mean HCC telomere length was 0.55, and normal liver controls exhibited an average telomere length value of 1.138.

Prediction of HCC Using Combined Markers

Logistic regression and receiver operating characteristics (ROC) curve analyses were performed to determine whether these assays discriminate HCC progressors from nonprogressors. Using logistic regression with 1000 bootstrap re-sampling for variable selection, $p53$ loss percentage and telomere length are the significant predictors that yield an area under the ROC of 0.929, a sensitivity of 87% and specificity of 81% with optimal choice of threshold (likelihood ratio chi-squared P<0.0001).

Conclusions

These studies demonstrate that molecular alterations detected in nontumorous cirrhotic liver can distinguish patients with HCC from those who are cancer-free with high sensitivity and specificity. Normal $p53$ prevents cells with genomic damage from entering the cell cycle, and if the damage is not repaired, activates apoptosis. Using $p53$ FISH, we detected increased $p53$ loss in progressors with HCC as compared to nonprogressors. It is not surprising that loss of this critical tumor suppressor function predisposes the organ to tumor formation.

We also detected telomere lengthening in progressors as compared to nonprogressors. In normal cells, telomeres shorten with each cell division until they become critically short, resulting in senescence, or are lengthened by the cellular enzyme telomerase. One potential mechanism for telomere lengthening in progressors is reactivation of telomerase; future studies will assess telomerase activity in these patients.

Using ROC analysis, optimal prediction of progressor versus nonprogressor status was obtained combining both biomarkers, $p53$ loss and telomere length. These data not only hold promise as improved biomarkers of HCC risk in chronic hepatitis but also provide important insights into the organ-wide genetic changes that accompany the development of HCC in HCV cirrhosis.

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References