Paroxysmal Nocturnal Hemoglobinuria

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Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal hematopoietic stem cell disorder with classical clinical manifestations of hemolytic anemia, bone marrow failure, and thrombosis. Additional manifestations include abdominal pain, renal failure or pulmonary hypertension. It is a rare condition primarily affecting adult men and women equally. The cellular defect of the disease involves glycophosphatidylinositol (GPI) anchor deficiency due to an acquired mutation in the gene involved in the first step of GPI anchor biosynthesis, phosphatidylinositol glycan class A, PIG-A.

Normally, GPI anchored proteins (GPI–AP) are synthesized in the endoplasmic reticulum through covalent attachment of a GPI anchor to a protein destined to be expressed in vesicles or on the cell surface. GPI–AP mediate a number of important cellular processes. In PNH, a somatic mutation arises in PIG–A of a hematopoietic stem cell often de novo or in the setting of aplastic anemia. PIG–A is located on chromosome Xp22.1 and codes for the synthesis of N-acetylglucosaminyl phosphatidylinositol. Various mutations throughout PIG–A have been identified in PNH cases, with most consisting of frameshift mutations resulting in a non–functional protein. All of the progeny of the hematopoietic stem cell clone, including red blood cells (RBC), white blood cells (WBC) and platelets, have a global decrease or absence of GPI–AP.

Two key GPI–AP absent or decreased on the progeny of PNH clones are decay accelerating factor (CD55) and membrane inhibitor of reactive lysis (CD59). CD55 and CD59 are negative regulators of the complement system and serve to protect host cells from damage by autologous complement. CD55 inhibits C3 and C5 convertases, whereas CD59 reduces the amount of membrane attack complex formed. Thus, decreased or
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absent levels of CD55 and CD59 increase the susceptibility of complement activity, resulting in inflammation, cell lysis, endothelial activation, platelet activation and promotion of a prothrombotic state. Cell-free hemoglobin released during complement mediated hemolysis scavenges and depletes the bioavailability of nitric oxide, manifesting with clinical symptoms and pathology. Hemolysis in PNH is chronic, yet can be triggered in response to inflammation, infection, or physiological stress (surgery). The factors contributing to the selected expansion of the PNH clone are incompletely understood; however, expansion of the clone further contributes to manifestations of the disease.

In conjunction with clinical and laboratory findings of hemolysis, flow cytometry has supplanted prior complement-mediated lysis assays, including the Ham test (acidified serum test) and sucrose hemolysis test, to facilitate diagnosis and monitoring of PNH. Flow cytometry should be performed in high-quality pathology laboratories and interpreted by experienced hematopathologists. Established guidelines for the use of flow cytometry in patients with clinical indications for PNH testing recommend evaluation of the absence or severe deficiency of GPI–AP on at least two cell lineages including neutrophils, monocytes and RBC with two independent flow cytometry reagents. A variety of antibodies to evaluate GPI–AP are available, including CD55, CD59, CD16, CD24, CD48, CD66b and CD157. A key reagent used in the direct evaluation of GPI anchor surface expression is the use of FLuorescent AERolysin (FLAER), a modified inactive variant of the bacterial aerolysin, which binds to GPI. FLAER is only used in the evaluation of WBC due to inadequate enzymatic processing on the RBC membrane. CD55 and CD59 are only used in the evaluation of RBC as they do not separate PNH clones adequately in granulocytes. Appropriate controls for each parameter are key to interpretation of the flow cytometry results and are institution dependent. Flow cytometry for PNH evaluation is one of the extensively controlled assays in the Northwestern Medicine’s flow cytometry laboratory.

In a patient with PNH, flow cytometry will demonstrate a population of cells with marked decrease or absence of all chosen GPI anchored antigens and FLAER. The patient with PNH may also have a population of cells expressing GPI–AP and FLAER as these did not originate from the PNH hematopoietic stem cell clone. An example of this is demonstrated in Figure 1. The percentage of PNH cells is reported for each respective lineage (granulocytes, monocytes and RBC). WBC are the preferred lineage to evaluate for the percentage of PNH cells as the percentage often differs with RBC and varies with clinical status. Although PNH is a rare disorder, given its clinical importance, clinicians need to maintain an elevated index of suspicion for this disease. This is particularly important in patients with hemolysis, thrombosis, and bone marrow failure. With still many unanswered questions and limited therapeutic options, further investigation of PNH is warranted.

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Figure 1: Flow cytometry histograms of a patient without and with PNH. Normal control patients express FLAER and CD157 on monocytes (A) and granulocytes (C). Patients with PNH show a population with absent staining for FLAER and CD157 in monocytes (B), and granulocytes (D). Normal control patients express a single population of RBC with CD55 and CD59 expression (E), whereas patients with PNH include a population of RBC with negative staining for CD55 and CD59 (F).
Diffuse gliomas are the most common primary brain tumor in adults, affecting ~20,000 people in the United States annually. The majority of gliomas are either astrocytic (~70%) or oligodendrogial (9%). Diffuse gliomas can be categorized by WHO grade: low-grade (WHO grade II), anaplastic (WHO grade III), and glioblastoma (GBM, WHO grade IV). GBMs are thought to arise through one of two main pathways. The first involves de novo development without a precursor lower grade lesion, termed “primary glioblastomas.” The second starts as a grade II or III lesion and progresses to glioblastoma over time, termed “secondary glioblastomas.” Secondary GBMs are relatively rare compared to primary GBMs, comprising ~5% of all GBMs. Secondary GBMs also typically affect younger patients (median age ~45 years) than do primary GBMs (median age ~60 years). Histologically, however, the two are indistinguishable.

Since 2008, several studies have revealed that the majority (~80%) of low-grade gliomas and secondary GBMs harbor mutations in isocitrate dehydrogenase (IDH) genes. In contrast, primary GBMs are largely negative for these mutations. IDH1 is the gene that is most commonly mutated but IDH2 mutations are also described. The mutations that occur in both genes are missense mutations, which typically cause a single amino acid change at arginine residue R132 in IDH1 and at R172 in IDH2. Both gene products function in the Kreb cycle and reduce the effects of oxidative stress through production of NADPH from NADP+ by catalyzing oxidative decarboxylation of isocitrate to alpha-ketoglutarate. IDH mutations affect the protein products’ active site, reducing its ability to bind isocitrate, leading to lower levels of alpha-ketoglutarate and NADPH, a cofactor important for reduction of glutathione and protection from reactive oxygen species. IDH mutations in gliomas are thought to be driver mutations, occurring early in the pathogenesis of these tumors, however, their exact mechanism of oncogenesis is not clear. Early studies suggested that the mutant proteins act in a dominant-negative manner through heterodimerization to and reduction in activity of wild-type IDH. More recently, it has been suggested that the mutant proteins convert alpha-ketoglutarate to R(-)-2-hydroxyglutarate, a compound that may act as an “oncometabolite.”

Generally, IDH mutations are considered independent good prognostic markers, with several studies describing longer survival in patients with IDH1 or 2–mutated grade II–IV gliomas than in those without IDH mutations. Some data suggest that IDH mutations influence responses to certain treatments, such as radiation and specific chemotherapeutic regimens as well. Additionally, early studies of selective IDH inhibitors have shown promising results and active research is still ongoing.

Detection of IDH mutations can be performed in several ways. A monoclonal antibody has been developed specifically against the mutated IDH1 protein derived from the most common IDH1 mutation (R132H), which can be used as an immunohistochemical stain on paraffin-embedded tissue. At NMH, if a diffuse glioma is negative for this stain, the paraffin-embedded tissue is sent for molecular testing, which is performed using a pyrosequencing assay. Briefly, the DNA from the tissue is extracted and the most commonly mutated segments in IDH1 (codon 132) and IDH2 (codons 140 and 172) are amplified by PCR using specific primers. These DNA segments are then sequenced by pyrosequencing, which is performed by addition of a single nucleotide at a time that, if incorporated into the growing sequence by DNA polymerase, releases a pyrophosphate that induces a chemical reaction resulting in a chemiluminescent signal detected by the machine. In this manner, the sequence of interest is determined and the presence of a point mutation can be identified.
IHD Mutations and MGMT Methylation in Brain Tumors
(contd.)

Epigenetic silencing of the gene encoding the enzyme O6-methylguanine-DNA methyltransferase (MGMT) through promoter methylation is another important molecular marker in glioblastomas. The MGMT protein is a highly evolutionarily conserved enzyme involved in DNA repair. Normally, DNA alkylating agents induce addition of alkyl adducts to guanine nucleotides, which leads to DNA base mismatching and double stranded breaks and subsequent cell death. MGMT removes these alkylating lesions, inhibiting DNA breakage and cell death from occurring. MGMT promoter methylation has been reported in ~45% of GBMs. It results in transcriptional silencing and inhibition of MGMT expression. MGMT promoter methylation is considered a strong and independent favorable prognostic marker in GBM patients receiving chemotherapy with alkylating agents. Patients with GBMs with methylated MGMT promoters have been shown to have significantly longer survival after treatment with the alkylating agent temozolomide than patients with unmethylated MGMT promoters. Additionally, it has been shown that elderly (>65–70 years) patients with GBMs lacking MGMT promoter methylation derive little benefit from such chemotherapy. The mechanism underlying this improved response to alkylating agents involves the reduced ability of tumor cells with decreased MGMT activity to reverse the effects of the therapy.

IDH mutations and MGMT promoter methylation show some relation in gliomas. In IDH mutated tumors, increased levels of R(−)-2-hydroxylutarate aberrantly produced by mutant IDH 1 or 2 enzymes leads to global changes in DNA methylation in the cell. Specifically, R(−)-2-hydroxylutarate inhibits various enzymes that leads to increased methylation and resultant inhibition of a number of genes, including MGMT. Therefore, MGMT promoter methylation is almost always present in IGH mutated gliomas. However, the reverse is not true (not all MGMT methylated tumors harbor IGH mutations).

At NMH, molecular testing for MGMT promoter methylation uses a bisulfite sequencing technique. The patient DNA is treated with sodium bisulfite, which converts unmethylated cytosine residues to uracil while methylated cytosines remain unchanged. After amplification of the promoter sequence by PCR, the sequence is determined using a pyrosequencing assay, similar to that used for IDH mutational analysis, where differences in methylated versus unmethylated DNA sequences can be identified. In addition, the degree of methylation can be quantified based on the ratio of different nucleotides added during sequence extension.

References:


Next-Generation Sequencing In the Diagnostic Molecular Biology Laboratory

By: Timothy Taxter, MD, Resident in Pathology

We always overestimate the changes that will occur in the next two years and underestimate the changes that will occur in the next ten – Bill Gates

With the acquisition of an Ion torrent PGM last year, Northwestern Memorial Hospital joined many other healthcare organizations making commitment to next generation sequencing (NGS) for personalized medicine. Although primarily used for oncology, this transition represents an exciting time for all disciplines of medicine. The 3 billion base pairs that make up the human genome dictate our ability to metabolize drugs, our propensity to develop cancer or Alzheimer’s disease, and maybe even our ability to perform differential equations [1]. When the human genome was first successfully sequenced on February 15th, 2001, through the joint efforts of the NIH led by Francis Collins and Celera Genomics led by Craig Venter, the biological world appeared to be at our finger tips.

The promise

In a way, biology is at our finger-tips. With the recent applications of Clustered regularly-interspaced short palindromic repeats / CRISPR-associated protein 9–nuclease (CRISPR/Cas9) for gene editing, the ability to re-engineer life is rapidly progressing, and sequencing technology is one of the core drivers of that process. The implications of these types of technologies are seemingly limitless. In the case of sequencing, the best example is the Encyclopedia of DNA Elements (ENCODE) project. In 2001 it was widely accepted that 98% of the human genome consisted of redundant non-functional DNA regions that happened to accumulate throughout our evolution. Sequencing along with other complimentary molecular technologies taught us this could not have been a more incorrect assumption. Our non-coding DNA represents an exquisitely complex regulatory network that provides precise control over our molecular machinery. This is exactly the type of thing Bill Gates was alluding to. The ENCODE study was launched in 2003 and by 2013 our entire understanding of the genome had changed.

The reality

Yet at the same time, NGS is far from ubiquitous in hospitals and clinical practice despite its widespread availability beginning with the Illumina’s release of their first bench-top sequencer MiSeq in 2011. The reason for this is a complex relationship between hospital financials and the clinical value that NGS adds to patient care. In 2011, the value of NGS compared to established molecular testing technologies was minimal given that at the time only a handful of mutations, such as EGFR in lung cancer, KRAS in colon cancer, and BRAF in melanoma,
Next-Generation Sequencing In the Diagnostic Molecular Biology Laboratory (contd.)

had clinical value. However, with the rapid mapping of the cancer genome and the explosion in the number of targeted therapeutics, conventional testing can no longer provide the scale currently required for personalized medicine. This evolution in clinical oncology is what leads to the implementation of NGS in the Diagnostic Molecular Biology (DMB) laboratory which utilizes the 22 gene Ion Ampliseq Colon and Lung panel to test for mutations associated with targeted therapies and resistance to therapy. For example, in non-small cell lung cancer the Ampliseq panel covers EGFR activating mutations such as L858R that can be targeted by tyrosine kinase inhibitors (TKI) and EGFR resistance mutations like T790M which confers resistance to TKIs. Currently, this panel provides a relatively comprehensive analysis of the genomic alterations that are clinically actionable, but for patients with advanced or metastatic cancer this level of sequencing is not sufficient to identify all the mutations with targeted therapies currently undergoing clinical trial. In the near future we’ll expand our testing to 150 or 300 gene panels to keep up with clinical demands.

The future

Looking towards the future we will have to keep pace with evolving NGS technologies and applications both in the near term and the long term. One example of a near term technology is the analysis of cell free tumor DNA (cfDNA) which is commonly performed by utilizing digital PCR coupled with NGS to monitor mutational allele frequency in the blood. This approach is rapidly becoming recognized as a transcendent approach to monitoring solid tumor patients in the post treatment setting for response to therapy based on the correlation between the mutational allele frequency and the outcomes and for the emergence of resistance mutations [2, 3]. Looking further into the future, more advanced sequencing methods like whole genome or RNA-seq for transcriptomic analysis may play an important role in patient care. RNA-seq in particular is an extremely powerful technology that can resolve differences in alternative transcription and splicing and shed light on protein isoform profiles. For instance, VEGF, commonly understood as a pro-angiogenetic gene, has multiple protein isoforms with both pro- and anti-angiogenesis activity. Understanding protein isoforms in a therapeutic context may have significant clinical ramifications for targeted treatment and predicting prognosis [4]. Of course, none of these possibilities are trivial challenges. The amount of data created with such methods increases exponentially, which requires integration of bioinformaticians and data scientists into molecular pathology labs. The DMB lab understands these trends and has added a bioinformatician to aid in the development of innovative analysis methods, database technologies, and electronic medical record applications. This commitment to informatics will be crucial to move forward because it will be our job to provide coherency and interpretation to this complex molecular data [5]. A perfect example of this at NMH is our department’s involvement with the newly established molecular tumor board through the Northwestern Medicine Therapeutic Institute (NMDTI) where we play a central role in the integration of advanced molecular diagnostics with clinical trial decision-making. The future of personalized medicine is molecular pathology.
Next-Generation Sequencing In the Diagnostic Molecular Biology Laboratory (contd.)

References:


Zika Virus: The 2015–2016 Outbreak

By: Julianne M. Ubago, MD, Resident in Pathology

Over the last several months, the Zika virus has gained worldwide attention due to the recent outbreak that has spread throughout much of the tropics of the Western Hemisphere. The Zika virus was originally discovered in 1947 in the Rhesus monkey population of the Zika forest in Africa and was first identified in humans in 1968 in Nigeria. It had not been identified in the Western Hemisphere until May 2015 when the current South American outbreak began in Brazil. As of February 10, 2016, there have been 52 reported cases in the US (3 in Illinois) from travelers returning from affected areas, but there have been no locally transmitted cases in the continental US. Currently there is local transmission of the virus in the US territories of Puerto Rico, the US Virgin Islands, and American Samoa.

Zika virus (ZIKV) is a single-stranded RNA flavivirus which is transmitted by the Aedes aegypti and Aedes albopictus mosquitos. These species also transmit the viruses responsible for Dengue Fever, Chikungunya, and Yellow Fever and the Zika virus often has a similar constellation of symptoms. It is usually a self–limited illness lasting for up to one week with symptoms of fever, headache, muscle pain/weakness, and maculopapular rash. Interestingly, only about 1 in 5 infected individuals exhibit symptoms; the remainder are asymptomatic. The disease is usually self–limited, but there have been recent concerns about neurologic sequelae and particularly a rise in cases of Guillain Barre Syndrome (GBS). While there has been an approximately 20% increase in GBS in Brazil over the last year, it is unclear whether this is specifically due to Zika virus or if it may be associated...
Zika Virus: The 2015-2016 Outbreak (contd.)

with an increasing incidence in Dengue and Chikungunya cases in the region as these viruses have also been associated with GBS.

The most concerning reports regarding Zika virus have been the possible association with microcephaly in fetuses whose mothers have reported Zika-like symptoms. There has been a 20-fold increase in microcephaly cases over the past year in Brazil. While much remains to be studied regarding this association, one of the first case reports published in the New England Journal of Medicine reported on a fetal autopsy case with microcephaly whose mother experienced symptoms at 13 weeks gestation. Zika virus was identified in the fetal brain tissue by RT-PCR, but not in any other fetal organs. Two other case reports have also identified Zika virus in the amniotic fluid of fetuses with microcephaly. Furthermore, Zika has been identified in other bodily fluids and there have been documented case of sexual transmission of the virus.

Zika virus can be identified in the blood of an infected individual for approximately 10 days after the onset of symptoms. It has also been reported in other bodily fluids such as semen for up to 10 weeks after an infection, long after it has cleared from the bloodstream. It is best identified in serum by RT-PCR and all testing is currently being done by the US Center for Disease Control (CDC). Thus, Zika virus RT-PCR is a send-out test at Northwestern with a 4-19 day turn-around time. Additional testing information can be found by searching “zika” in the online Pathology Handbook on the NMI website. The only treatment is supportive care and no vaccines or medications currently exist to treat or prevent infection. The CDC recommends strict measures to prevent against mosquito bites. They also recommend that all pregnant women or women planning to become pregnant avoid travel to outbreak areas and abstain from sexual intercourse with partners who have recently traveled to affected areas. Currently testing at Northwestern is being done on any patients with known exposure who are experiencing symptoms concerning for Zika virus.

References:


Rapid Microorganism Identification using MALDI Technology

By: Mike Malczynski, Operations Coordinator in Clinical Microbiology Laboratory

In October 2014, the Microbiology Laboratory began using a newly FDA approved instrument based on matrix assisted laser desorption ionization–time of flight (MALDI–TOF) technology to identify microorganisms. MALDI –TOF is replacing conventional phenotypic identifications for most bacterial and fungal strains. Once the organism is recovered by culture from a clinical specimen, it takes only a few minutes to have a correct identification. In addition, some antibiotic resistance and bacterial toxins may be detected.

Matrix-assisted laser desorption ionization is a soft ionization technique allowing large molecules such as proteins to remain relatively intact during the ionization process. Identifying a microorganism with MALDI–TOF involves the following steps. First, the growing colonies are spotted on a target plate and covered with the matrix to crystalize. The matrix "buffers" the sample, preventing its decomposition, and enabling transformation of laser light into heat. Next, a laser is applied. The matrix absorbs energy from the laser, releasing it into the cells as heat. This causes the cellular proteins to desorb and form singly charged ions. Then the ions are separated in a vacuum chamber. The lighter ions travel faster and are consequently detected earlier than the heavier ions. The mass-to-charge is plotted against signal intensity and this serves as the fingerprint for identification. Ribosomal proteins are mostly useful for bacterial and fungal identification.

The MALDI has been incorporated into routine use for most bacterial and fungal identifications in the Clinical Microbiology Laboratory at Northwestern. The test requires colonies recovered from culture. Normally this occurs within 18–24 hours after plating the patient specimen to media, but for some fungal and fastidious microorganisms culture growth can take longer. Unfortunately, the instrument cannot be used on direct patient specimens, as the bioburden is too low for the instrument to provide accurate detection levels.

The advantage of this new technology is to provide rapid identifications once microorganisms are recovered from culture. Prior to using the MALDI instrument, a microorganism identification was based on phenotypic tests, including Gram staining, growth characteristics, and biochemical patterns. Most microorganisms were given an identification within 6–24 hours after setting up these tests. Using the MALDI, the same microorganism identifications are provided in a matter of minutes. Having the identification of organisms in a more timely fashion allows clinicians to provide targeted antimicrobial treatment, or to discontinue treatment should the organism be deemed non-pathogenic. With an increasing emphasis on properly managed antimicrobial use not only within the hospital but nationally, instruments such as the MALDI can provide clinicians with another useful tool to better manage patient care and treatment.
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