Review Article

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A Review of Recent Advances in Cytopathology for Diagnosis of Hepato-Biliary Lesions

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ABSTRACT

Cytology has emerged as an important diagnostic modality for hepato-biliary lesions because of its minimally invasive, rapid turnaround time and cost-effective nature. This is especially important in diagnosis of neoplastic pathology of liver, gall bladder or bile duct where biopsy is difficult and delayed diagnosis may lead to complications or even mortality. The advent of newer molecular diagnostic modalities like polymerase chain reaction (PCR), fluorescent in situ hybridization (FISH), immunochemistry or more recently circulating tumour cells and next generation sequencing (NGS) have not only led to precise diagnosis but are also helpful in targeted therapy and prognostication of tumour. Cytology which is a minimally invasive technique can be judiciously used for advanced molecular analysis for diagnosis and management of hepato-biliary lesions. Awareness regarding the use of cytological samples for molecular analysis in hepato- biliary lesions is essential so that mutational changes may be easily determined without undergoing any invasive technique in these deep-seated lesions. It is also necessary that guidelines on molecular cytology should be chalked out so that uniform reporting is followed with maintenance of high-quality standards.

Keywords: *Cytology, Hepato-Biliary Lesions, Molecular Techniques*

Introduction

Cytology has emerged as an important diagnostic modality for hepato-biliary lesions because of its minimally invasive, rapid turnaround time and cost-effective nature. This is useful for planning of management and hasten the therapy to prevent further complications. Image guided fine needle aspiration cytology (FNAC) is being increasingly used for diagnosis of inaccessible deep seated hepato-biliary lesions with collaboration of experienced radiologists and pathologists. The image guided systems may include percutaneous computed tomographic (CT), ultrasound guidance (USG) or more recently endoscopic ultrasound guidance (EUS) [1,2,3]. The adequate cytological material so obtained is interpreted in light of clinical and radiological findings leading to definite diagnosis. This is especially important in diagnosis of neoplastic pathology of liver, gall bladder or bile duct where biopsy is difficult and delayed diagnosis may lead to complications or even mortality. The Papanicolaou Society of Cytopathology (PSC) has also provided guidelines for the interpretation and reporting of cytologic specimens of pancreatico-biliary lesions [4]. These guidelines address indications, techniques, terminology and nomenclature, ancillary studies, and post procedure management of these pancreatico-biliary lesions [4].

The advent of newer molecular diagnostic modalities like polymerase chain reaction (PCR), fluorescent in situ hybridization (FISH), immunochemistry or more recently circulating tumour cells and next generation sequencing (NGS) have not only led to precise diagnosis but are also helpful in targeted therapy and prognostication of tumour. Thus, for understanding the pathogenesis and precise diagnosis the morphological changes should be supported by molecular analysis for cytological diagnostic work up of hepatic biliary lesions. It has also been observed that cytological material may be more useful than formalin fixed paraffin sections for molecular analysis as degradation of nucleic acids is minimal [5]. It is also essential that each laboratory should validate minimal tumour cell percentage or cellularity to perform cytopathological molecular testing. It has been reported that >20% tumour cellularity are required for Sanger sequencing, >10% cellularity for PCR based assays and >5% tumour cellularity for next generation sequencing [5]. Recently, it has been reported that stained cytological smears can also be appropriately used for FISH analysis by simply destaining them in acid alcohol solution and thus leading to judicious use of cytological material. Thus the role of molecular diagnostic techniques in cytopathology especially in hepato- biliary neoplasms is promising.

Papanicolaou Society of Cytopathology guidelines for the interpretation and reporting of cytologic specimens of pancreaticobiliary lesions

Techniques for Cytologic Sampling of Pancreatic and Bile Duct Lesions

Brushing cytology is considered to be adequate technique for obtaining cytological material for diagnosis of these lesions. It has been reported that this technique yiels better results than bile aspiration alone and provides more accurate diagnosis than focal biopsy [6]. Endoscopic ultrasound and fine-needle aspiration (EUS-FNA) is now considered technique of choice for diagnosis of pancreatic malignancies.

Proposed Pancreatobiliary Terminology Classification Scheme [4]

The Papanicolaou society has proposed cytological categories for diagnosis of pancreatico-biliary lesions which include six categories. They are non-diagnostic (category I), negative for malignancy (category II), atypical (category III), benign or other neoplasm (category IV), suspicious for malignancy (category V) and positive for malignancy (category VI) [4]. Category III is heterogenous category which include cases with reactive changes, low cellularity and dysplastic cells. The cellular morphology in this category show atypia which are more than reactive atypia but insufficient to call malignant or suspicious of malignancy. Category IVa which includes benign neoplasms includes serous cystadenoma of pancreas. The smears are cellular and representative of pancreatic benign neoplasms with or without clinico-radiological correlation. Category IVb is a controversial category which includes pre-malignant (intraductal papillary mucinous neoplasm or mucinous cystic neoplasm) and low-grade malignant neoplasms of pancreas (solid pseudopapillary neoplasms or pancreatic neuroendocrine tumors). The rationale for this category is relatively conservative management of most of the lesions. Category V shows cytological smears which have strong suspicion for malignancy but the cytological material is either scant or qualitatively insufficient for a conclusive diagnosis of malignancy. It has been reported that risk of malignancy in such cases is 80-96% [7]. Category VI includes cases which frankly display malignant cytological features and includes ductal adenocarcinoma, cholangiocarcinoma, acinar cell carcinoma, high-grade neuroendocrine carcinoma (small cell and large cell), pancreatoblastoma, lymphomas, sarcomas, and metastases to the pancreas.

Selection of tissue specimen type for molecular Cytopathology

Before subjecting the tissue for molecular testing, it is essential to have knowledge of selection of cytopathological tissue for molecular analysis. The fresh aspirated cells from FNAC are better for DNA and RNA isolation while paraffin embedded cell blocks may degrade the RNA during processing and embedding and are thus not usually good for its isolation [5]. Therefore, cell blocks should be usually avoided if RNA has to isolated especially for hepatic infections molecular analysis. During preparation of cell blocks, prolonged fixation in formalin may cause damage in DNA leading to false mutational impression [8]. It is therefore recommended that formalin fixation should be less than 72 hours for optimal DNA extraction [9]. Freshly aspirated cells fixed in ethanol or methanol are considered best for preserving DNA and RNA with minimal nucleic acid changes [10]. It is also important to assess the cellularity of cytological material sent for molecular analysis in hepato-biliary lesions. In cases with low cellularity certain pre analytical and analytical target cell enrichment techniques may be used which include laser or manual micro dissection and reduce normal tissue component. Laser capture microdissection (LCM) isolates cells of interest by using laser which may be further subjected to molecular analysis. It has been judiciously used to study various liver diseases from DNA and RNA sequencing to mass spectrometry [11]. Nested PCR approach may also be used to enrich targeted nuclei acids and molecular diagnosis.

Molecular techniques applied for diagnosing hepato-biliary lesions

PCR based molecular assays

PCR which involves amplification of nucleic acids is an important diagnostic modality for not only infectious pathology in liver, gall bladder or bile duct but also to diagnose mutations in neoplasms. It is widely available and is comparatively cheap in comparison of other molecular techniques. In addition to classic PCR, other methods have also been used in cytological samples including real time PCR, high-resolution melting analysis (HRMA), restriction fragment length analysis (RFLP), COLD (co-amplification at lower denaturation temperature)-PCR, scorpion amplification refractory mutation system (S/ARMS), and peptide nucleic acid- locked PCR (PNA-LCA PCR) [5]. These modifications have increased sensitivity and specificity than classic PCR. Real time PCR also plays an important role in diagnosis of occult hepatitis B infection (OBI) which is defined as negative hepatitis B surface antigen, positive/negative anti-hepatitis B core antibody and hepatitis B virus (HBV) DNA detectable in

serum or liver tissue. Its pathogenesis is due to sG145R mutation in the HBsAg gene leading to low binding affinity to monoclonal antibody against HBsAg or interference with the splicing of S gene mRNA by substitution of G-to-A at position 458 of the surface gene leading to lack of HBsAg expression and low replication of HBV DNA [12]. Cayarga *et al* have observed in their study that fast real time PCR assay for hepatitis B virus DNA quantification has 100% specificity and may be used in diagnosing HBV infection and monitoring drug response [13]. HRMA is a rapid and cost-effective method that uses combination of real time PCR and evaluation of DNA melting curves to accurately detect mutations by comparing the patterns of obtained curves to preset curves from non-mutated sample [5]. It is a sensitive, multipurpose approach for diagnosis and epidemiological investigations of parasitic infections especially plasmodium, leishmania or other protozoa in liver or bile duct [14]. Apart from infections, PCR based methods are also useful in diagnosing the neoplasms and mutations for management of tumours of liver, gall bladder and bile duct. Mu et al developed a multiplex quantitative polymerase chain reaction (qPCR) assay for detection of long noncoding RNA in hepatocellular carcinoma (HCC) [15]. Multiplex mutational analysis which consists of primer sets targeting multiple genes is useful on cytological material. The DNA extracted from fresh cells obtained by FNA subjected to multiplex mutation analysis show results similar to frozen samples or higher than paraffin embedded tissue thus concluding that nucleic acids obtained from cytological samples are suitable for multiplex mutational analysis [5]. KRAS, p16INK 4A, TP53 mutations have been studied in gall bladder carcinoma using PCR-RFLP or other PCR based assays [16].

FISH based assays

It is the molecular technique which uses fluorescent labelled probes that binds or hybridises to the complimentary nucleic acid sequence in the tested sample leading to the diagnosis. It has been increasingly used in cytopathological specimens to detect chromosome number, amplifications, deletions, translocations and sequence of genes [17]. FGF19 amplification which is considered to be responsible for hepatic carcinogenesis and poor prognosis of HCC is detected by FISH [18]. This method is also considered useful in imaging hepatitis B virus (HBV) nucleic acids in cell culture [19]. Apart from this, FISH may also play an important role in increasing sensitivity of brush cytology of diagnosing cholangiocarcinoma in indeterminate biliary strictures [20]. Kushnir *et al* have also concluded in their study that PCR based mutational profiling and FISH are complimentary molecular tests that can significantly increase detection of biliary malignancies when used in combination with routine cytology of standard biliary brush specimens [21]. However, important limitation of FISH is high cost and therefore may not be feasible in resource limited settings. In addition, it can detect only limited number of alterations that can be identified simultaneously.

Immunocytochemistry

It is also an important molecular diagnostic method which can be easily used on cytological material both on methanol fixed fresh FNAC smears or cell blocks. This is especially useful to differentiate hepatocellular carcinoma from metastatic carcinoma of unknown primary. Hep Par-1 and Arginase-1 have 85-95% specificity to confirm hepatocytic differentiation of the neoplasm while panel of immunochemical markers like Glypican 3, heat shock protein 70, beta catenin, CD34 may be used to differentiate hepatic adenoma, hyper plastic nodule or HCC [22]. Recently over expression of HER2neu have been observed in gall bladder adenocarcinoma and it has been considered that it may be explored as potential therapeutic target [23].

Circulating tumour cells (CTC)

CTC are the hall mark of liquid biopsy which analyses tumour cells from non-solid tissue such as blood or body fluids. In hepatobiliary neoplasms body fluids like ascitic fluid may be considered as an important tissue sample to analyze CTC. This novel biomarker has been considered to play an important role in early detection, treatment and prognostication of HCC [24]. It has been reported that aberrant promoter methylation in circulating tumour DNA may be evaluated as screening tool for HCC [24]. Liquid biopsy also has promising role in screening and detection of cholangiocarcinoma and bile miRNA have been studied for its diagnostic utility [25].

Next generation sequencing (NGS)

Next generation sequencing may be applied to cytological specimens for mutational profiling of many genes on different types of tumours. This technique may be helpful in targeted therapies against mutational alterations in HCC. Harding et al have concluded that linking NGS to routine clinical care in HCC has the potential to identify patients which are likely to benefit from standard systemic therapies or to genome-directed targeted therapies [26].

Although all the above-mentioned methods may be important for precise diagnosis, prognosis and management of hepato-biliary lesions on cytological samples but financial burden may limit their use especially in resource limiting settings. In addition, lack of uniform reporting guidelines on cytology and ethical issues especially on NGS should also be tackled so that interpretation and management bias may be avoided.

Conclusion

Cytology which is a minimally invasive technique can be judiciously used for advanced molecular analysis for diagnosis and management of hepato-biliary lesions. The procured cytological samples are not only sufficient to determine the prognosis of neoplasms but may also be helpful for designing targeted therapies against them. Awareness regarding the use of cytological samples for molecular analysis in hepato- biliary lesions is essential so that mutational changes may be easily determined without undergoing any invasive technique in these deep-seated lesions. It is also necessary that guidelines on molecular cytology should be chalked out so that uniform reporting is followed with maintenance of high-quality standards.

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