Discussion

Pancreatic cancer is a growing concern in developed countries as deaths due to this condition are on the rise and the survival rate is less than 5%. Routine cancer detection techniques such as Computed Tomography (CT) scan and Magnetic Resonance Imaging (MRI) are not very useful in the identification and staging of pancreatic cancer.[1] Delayed diagnosis of pancreatic cancer in a large number of cases is the primary cause of its high mortality. The past few years have seen a number of efforts to improve the diagnostic accuracy of pancreatic cancer by making use of molecular markers and ultrasonography.[2] The main objective of this research has been to evaluate the recent diagnostic strategies in the field of pancreatic cancer such as EUS-FNA (Endoscopic Ultrasound – Fine Needle Aspiration) and molecular analyses using *K-RAS*, mucins and micro-RNAs by undertaking an extensive review of literature. The results obtained have been categorized as described below.

EUS-FNA

Endoscopic ultrasonography (EUS) has become a very important tool in the differentiation of pancreatic lesions. Its accuracy has been improved by EUS-guided fine needle aspiration, which allows an investigator to obtain a fluid sample and further characterize the lesion.[3] Samples obtained by EUS-FNA are often used for molecular analyses like *K-RAS* mutation analysis and mucin expression analysis.[4]

The value of *K-RAS* mutation analysis from EUS-FNA samples has been documented in a number of studies.[5,6,7] Analyses using EUS-FNA show no false positives and a very low rate of false negatives.[5,6] In the analysis of *K-RAS* mutations, samples obtained by EUS-FNA are highly specific for methylation markers and quite sensitive for the detection of mutations.[5] This might

be attributed to the fact that samples obtained by EUS-FNA show an absence of PanIN lesions, which serves to improve the diagnostic accuracy. Furthermore, samples from EUS-FNA are useful not only for the diagnosis of pancreatic cancers, but also other diseases such as chronic pancreatitis and autoimmune pancreatitis.[6] EUS-FNA is also useful in cases where cytopathologic analyses have proved inconclusive in the diagnosis of pancreatic cancer and a combination of cytopathologic analyses and *K-RAS* mutation analysis on EUS-FNA samples improves the diagnostic accuracy. However, one disadvantage of using EUS-FNA samples is that well-vascularized tumors may coagulate within the sample leading to difficulty in analysis.[7] As seen in Table 1, the specificity of detection of mutations within *K-RAS* from EUS-FNA samples is close to 100%, whereas the sensitivity ranges from 60 – 90%. Also, the accuracy is 70 – 80% with a positive predictive value of close to 100%.[5,6,7]

The collection of samples by EUS-FNA is also found to be useful for mucin expression analysis.[8,9] EUS-FNA is the technique of choice due to the fact that it is a noninvasive method and hence, high-quality samples are obtained for analysis. For detection of cancer-specific mucins in EUS-FNA samples, the sensitivity is 78 – 94% with a false negative rate of 3 – 5%. However, if the sample collected is inadequate, diagnosis based on mucin expression may not be accurate.[8] Analysis of mucin expression is particularly important for pancreatic cancers and mucinous neoplasms and the tumor-specific expression of mucins in EUS-FNA samples has been extensively characterized.[9]

The usefulness of EUS-FNA samples has been increased by using a micro-RNA-based approach in the diagnosis of pancreatic ductal adenocarcinoma. Again, this method will not be accurate if the sample collected is inadequate or contaminated. Fine needle aspirates collected without the guidance of EUS show a high rate of technical failure and lower sensitivity. Hence, for a higher

accuracy in diagnosis, use of EUS-FNA is strongly recommended.[10] As shown in Table 5, the diagnostic accuracy of both benign and malignant pancreatic cancers is much higher with the use of molecular analyses than by cytology alone, and this includes analysis of *K-RAS* mutations, expressions of mucins, and analysis of micro-RNAs.

Analysis of *K-RAS* mutations

K-RAS is an oncogene that has been demonstrated to get activated in pancreatic cancers due to certain mutations in the sequence. Most of the tumor-causing mutations are localized to codon 12 and hence, it is quite easy to detect mutations in this gene.[11] PCR-based techniques are usually used for the detection of these mutations due to their high sensitivity, reliability and ease of use.[12]

As seen in Table 1, studies have reported a high sensitivity of *K-RAS* mutation detection in EUS-FNA samples, which is in accordance with reports from European populations.[5] The use of EUS-FNA samples for the purpose of *K-RAS* mutation analysis improves the diagnostic value of the sample collection technique.[6] The advantage of *K-RAS* mutations is that it is present only in malignant samples, which improves the diagnostic accuracy and allows staging of the cancer.[5]

There is quite strong evidence regarding the combined use of *K-RAS* mutation detection and cytopathology in EUS-FNA samples for the detection of malignant pancreatic cancers.[7] As seen in Table 1, the combination of *K-RAS* mutation detection and cytopathologic analyses shows a significant increase in sensitivity, accuracy and negative predictive value as compared to *K-RAS* mutation detection alone or cytopathology alone. As cytopathology turns out inconclusive in a considerable number of samples, the coupling of *K-RAS* mutation analyses with cytopathology can help increase the diagnostic value of the samples.[7] The combination of *K-RAS* mutation

detection with detection of mutations in another gene, such as GNAS, can also help increase the specificity of detecting pancreatic cancers.[13]

The techniques most commonly used for analyzing *K-RAS* mutations are restriction fragment length polymorphism (RFLP) and direct sequencing; however, in order to improve the accuracy of the testing, Cycleave PCR has also been used and found to be more sensitive when compared to the other methods.[6] Another report has suggested the use of qPCR for *K-RAS* mutation detection as it is faster and less expensive as compared to RFLP.[7]

Mucin expression analysis

Mucins are glycoproteins that are either secreted by the epithelia or remain attached to the cell membranes, and the most important members include MUC2, MUC5AC, MUC5B, MUC6, and MUC19. The mucins that have been implicated in pancreatic cancer include MUC5AC, MUC1 and MUC4.[14] Different stages of pancreatic cancer show different patterns of mucin expression, for example, increase in expression of MUC1, neoexpression of MUC4, and overexpression of MUC17.[15] Analysis of the mucin expression profile of a sample can help not only in diagnosis, but also estimating the stage of the cancer.

An important category of pancreatic cancers include mucinous cystic neoplasms which are lined by mucin-secreting epithelial cells. A very direct and reliable way to identify these tumors is by mucin expression profiling by immunohistochemical staining. The presence of invasive disease is usually indicated by the presence of MUC1, while MUC2 and MUC5AC point towards noninvasive disease. These mucins also play a role in full-blown cancers and hold high diagnostic value.[16]

A study by Horn et al. found that positive samples obtained by EUS-FNA showed increased levels of MUC4 and MUC16 with a specificity of 100% and a sensitivity of 60 – 75%. MUC4 has been reported to show a gradual increase in its expression with the progression of pancreatic cancer, and it reaches its maximum expression in completely differentiated cancers. MUC16 has a higher sensitivity than MUC4 and hence, it is a suitable marker for detecting pancreatic cancers.[8] The comparison for MUC4 and MUC16 can be found in Table 4, which shows 100% specificity for both the markers; however, the sensitivity for both is highly variable.

Another study by Wang et al tested the diagnostic value of three other mucins – MUC1, MUC2, and MUC5AC – and found them to be valuable biomarkers for pancreatic cancer. The involvement of these mucins in breast, ovarian and gastrointestinal cancers has already been established, and now it is also linked to pancreatic cancers. Among these three mucins, MUC1 and MUC5AC show a significant increase in expression in pancreatic cancers, and their detection in EUS-FNA samples is relatively easy and reliable.[9] As seen in Table 2, MUC1 and MUC5AC are overexpressed only in malignant cancers and not benign cancers and hence, this has diagnostic value for well-differentiated cancers. Also, Table 3 shows an analysis of the diagnostic accuracy of mucin expression analysis with or without cytopathologic analyses. In the case of both MUC1 and MUC5AC, there is a significant increase in specificity, sensitivity and accuracy when mucin expression analysis is combined with cytopathology.

There are a number of reports in the literature that validate the use of mucin expression profiling in the diagnosis of pancreatic cancer and that describe specific changes in mucin expression at different stages of cancer. Other mucins that have been linked to pancreatic cancer include MUC6, which shows an expression profile similar to that of MUC5AC and could be an important tumor marker.[17] MUC7 expression has strongly been linked to pancreatic

adenocarcinoma and chronic pancreatitis, and could serve as a valuable marker for malignant cases.[18]

Micro-RNA based approaches

Micro-RNAs are small stretches of RNA molecules comprising of 19 – 25 nucleotides, which are highly stable and play important roles in transcription regulation. The advantage of micro-RNAs is that they can be isolated from samples of very low quality. The involvement of micro-RNAs in pancreatic cancer is quite complicated, for example, the production of miR-21 is increased in the presence of *K-RAS* gene product. During the early stages of pancreatic cancer, there is an increase in the production of miR-21, miR-221, miR-222 and let-7a, and a decrease in the production of miR-148. Hence, of all the techniques, the analysis of micro-RNA is the only method by which early stages of pancreatic cancer can be identified in EUS-FNA samples.[19]

A study by Brand et al. aimed to design a micro-RNA expression profile that could help in the diagnosis of pancreatic cancer. The micro-RNAs used were miR-24, miR-130b, miR-135b, miR-148a and miR-196. The specificity of detection of pancreatic cancer was found to be 90% as compared to 78% with cytopathologic analysis. Also, the sensitivity and positive predictive values were quite high indicating that micro-RNA based analyses is an attractive option in the diagnosis of pancreatic cancer.[10]

There have also been a number of other studies validating the use of micro-RNAs in detection studies. A study by Wang et al also documented the usefulness of micro-RNA based studies in the detection of early stages of pancreatic cancer.[20] Another study by Panarelli et al. used qRT-PCR and microarray analysis to study the expression of micro-RNAs in specimens obtained by EUS-FNA. This study confirmed the overexpression of miR-21 and miR-221 in pancreatic

cancer samples, and also identified the overexpression of other micro-RNAs which included miR-155, miR-100, miR-181b and miR-196a.[21] Differences in the micro-RNA expression profiles in benign and malignant pancreatic tissues have also been documented. The use of miR-196a and miR-217 particularly helps in distinguishing between benign and malignant cancers, and chronic pancreatitis.[1]

There are a number of methods for the analysis of micro-RNAs in EUS-FNA samples and there is no one standard method for doing so. Some of them include fluorescence in situ hybridization using labeled probes against specific micro-RNAs, immunostaining, and qRT-PCR.[22] There are at least 78 known micro-RNAs which show differential expression at different stages of pancreatic cancer. A number of studies have been undertaken to identify different combinations of these 78 micro-RNAs that would be most useful in the detection of different stages of pancreatic cancer. It has also been proved that micro-RNA analyses coupled with cytopathology has a higher specificity and sensitivity than micro-RNA analyses alone.[23] However, in spite of all the efforts, the use of micro-RNA analyses in EUS-FNA specimens for diagnostic purposes is still in its initial stages and it needs to be standardized to give an easy-to-use reliable protocol at the clinical level.

In summary, the importance of the use of EUS-FNA samples in the diagnosis of pancreatic cancers is increasing and a number of studies have reported different ways in which these samples are being used for diagnosis in the clinical scenario. These approaches include analysis of mutations in *K-RAS* gene, analysis of mucin expression profiles and analysis of micro-RNA expression profiles. It has been proved that these methods have more diagnostic value and a higher specificity and sensitivity when they are coupled with cytopathologic analysis. The use of one or more of these approaches depends on the availability of skilled manpower and

instrumentation; however, the regular use of these techniques is hoped to significantly lower the mortality rate of pancreatic cancer.

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