Two-photon Excited Fluorescence Imaging of the Pancreatic Solid Pseudopapillary Tumor without Hematoxylin and Eosin Stains

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Summary: Solid pseudopapillary tumor (SPT) of the pancreas is an epithelial tumor with low-grade malignant potential and present more common in females. At present, the gold standard for accurate diagnosis of pancreatic tumor was mostly depending on the pathological and/or cytological evaluation. In this work, TPEF microscopy was applied to obtain the images of human normal pancreas and SPT of the pancreas without hematoxylin and eosin (H&E) staining, for the purpose of identifying the organization structural, cell morphological, and cytoplasm changing, which were then compared to their corresponding H&E stained histopathological results. Our results showed that high-resolution TPEF imaging of the pancreatic SPT can clearly distinguish the pathological features from normal pancreas in unstained histological sections, and the results are consistent with the histological results. Moreover, we measured the nuclear-cytoplasmic ratios of the pancreatic SPT and normal pancreas to characterize their difference in the cytomorphological feature. It indicated that this technique can achieve the consistent information of pathological diagnosis, and has the potential to substantially improve the optical diagnosis and treatment of the pancreatic SPT without H&E staining in the future. SCANNING 38:245–250, 2016. © 2015 Wiley Periodicals, Inc.

Key words: two-photon excited fluorescence imaging, optical diagnosis, pancreatic solid pseudopapillary tumor, unstained histological sections

Introduction

Solid pseudopapillary tumors (SPTs) of the pancreas that presenting predominantly in young female patients are the neoplasms of low malignant potential in exocrine pancreas (Lee et al., 2008; Yagcı et al., 2013; Mahida et al., 2015). In accordance with the 2000 World Health Organization (WHO) classification system, pancreatic SPTs are usually defined as epithelial tumors composed of monomorphic cells surrounding delicate blood vessels and forming a solid mass as well as pseudopapillary structures, frequently showing hemorrhage, and cystic degeneration (Coleman et al., 2003; Wang et al., 2014). Approximately 75–80% of SPT of the pancreas is developed from the pancreas, but may occasionally be invasions of the adjacent organs or distant metastasis, such as the liver, lymph node mesentery, omentum, and peritoneum (Papavramidis and Papavramidis, 2005; Yin et al., 2012). The patient usually shows nonspecific clinical symptoms (Sperti et al., 2008). Pain and abdominal mass or fullness is reported as most common symptoms, and less frequent symptoms include jaundice, gastrointestinal obstruction, anaemia, and pancreatitis (Ghimire et al., 2014). Due to the uncertainty of the origin and nonspecific clinical symptoms, SPTs of pancreas are often misdiagnosed (Tajima et al., 2012). On the pathology, if this kind of tumors turns up perineural or vascular invasion with deep invasion of the surrounding tissue or distant metastases, SPT of the pancreas should be potentially malignant and developing metastatic disease (Goh et al., 2007; Yang et al., 2008).
Surgical resection is the first choice of treatment for SPTs (Yagcı et al., 2013). It was reported that 95% of cases will be cured by complete ectomy (Yao et al., 2011). Making early diagnostic markers is vitally important to understanding the fundamental changes in SPTs of the pancreas, which will help to improve the accuracy of diagnosis before surgery.

The major histological diagnosis of pancreatic SPTs was mostly depending on the traditional H&E staining, which has several disadvantages such as time-consuming, environmental pollution by dyestuff, and over-or under-staining. Consequently, developing a rapid and pollution-free diagnostic imaging tool that obtains images of comparable resolution and quality as standard H&E-stained images is important for earlier identification and treatment of SPTs of the pancreas. Recently, two-photon excited fluorescence (TPEF) microscopy has been applied to image tissue architecture and cellular morphology of unstained tissue samples in biomedical research (Chen et al., 2014a). This technique presents a non-invasive and high-resolution laser scanning enabling visualization of cellular and subcellular structures without any exogenous fluorescent agents while maintaining tissue viability (Zipfel et al., 2003; Wilder et al., 2004; Huang et al., 2008). Compared with traditional optical imaging, TPEF microscopy has the advantages of deeper penetration depth, higher resolution, lower photo-bleaching, and photo-toxicity. Most importantly, the TPEF signal generating from intrinsic molecular signals without the need for fluorochrome staining in biological tissues can be used to clearly visualize cell morphology. In this article, we attempt to achieve the pathological information by using TPEF imaging and compare the TPEF images with the H&E histological results in order to demonstrate that TPEF microscopy has the potential to diagnose SPTs of the pancreas without H&E staining.

Materials and Methods

Sample Preparation

A 27-year-old woman had been diagnosed with SPT in the tail of the pancreas. The SPT sample was resected from the patient in the First Affiliated Hospital of Fujian Medical University (Fuzhou, China) in September, 2012, as well as the normal pancreatic tissue excised 2 cm beyond the resection margin. After being removed by surgeons, the specimen was disposed into a paraffin block based on a standard pathologic procedure, which is included (10%) fixation, alcohol dehydration, and paraffin embedding. Before TPEF microscopic imaging, two consecutive sections of each sample were cut in 5 μm thick by cryostat microtome and deparaffinized by alcohol and xylene. One of the sections was used for MPM imaging, and the other one was stained with H&E for histological images and examined by standard light microscopy. TPEF images and H&E stained images were compared and analyzed by the same attending pathologist. The protocol for the research project has been performed with the informed consent of this patient and approved by the Institutional Review Board of the First Affiliated Hospital of Fujian Medical University.

TPEF Microscopic Examination

The TPEF microscopic system used in this work has been described previously (Chen et al., 2013; Xu et al., 2013). In short, the system was built based on three basic components: an inverted microscope (LSM 510META, Carl Zeiss), a mode-locked Ti: sapphire laser tuning range of 700–980 nm (Mira 900-F, Coherent), and a high-throughput scanning inverted microscope (Zeiss Axiovert 200). A 10W solid-state laser (Verdi-10, Coherent) was used to generate pulse widths of approximately 130 fs at a repetition rate of 76 MHz and the laser intensity was controlled by using an acousto-optic modulator. In this work, an excitation wavelength of 810 nm was used and a Plan-Neofluar objective (40×, NA = 0.75, Carl Zeiss) was employed for focusing the excitation beam into tissue specimens and collecting the backward intrinsic TPEF signals in the wavelength range from 350 to 710 nm. Large-area images were obtained by using an optional HRZ 200 fine-focusing stage (HRZ 200, Carl Zeiss). All images have a 12-bit pixel depth. The images were obtained at 2.56 μs per pixel.

A light microscope (Eclipse Ci-L, Nikon, Japan) with a CCD (DS-Fi2, Nikon, Japan) was used to image H&E stained sections. A Plan-fluor objective (40×, N = 0.75, Nikon, Japan) was employed for histological examination.

Quantification Methods

The nuclear-cytoplasmic (N/C) ratio was defined as the size of the nucleus relative to the area of the cytoplasm within cells in each tissue, to quantify the different cytomorphological features between the pancreatic SPT and normal pancreas. 20 cells with cytoplasm and nuclei demonstrating complete and clearly discernible outlines were selected in each TPEF or H&E stained image. And then the cells were circled and measured by using an optional fine-focusing stage (HRZ 200, Carl Zeiss). Quantitative data were expressed as the mean value followed by its standard deviation, shown as “mean ± SD.” The one-way ANOVA and least-significant difference analyses were performed to determine the statistically significant differences in the N/C ratios between normal and tumor tissues.
Statistical analyses were performed using SPSS software (version 16.0). Differences were considered to be statistically significant when the p values were less than 0.05.

**Results**

**TPEF Microscopic Imaging Features of the Normal Pancreatic Tissue**

The nonlinear optical image of human normal pancreatic tissue clearly demonstrates regular tissue architecture and cell morphology (Fig. 1A), as indicated by the TPEF signal. It is clearly identified that the bright fibrous connective tissue (white triangle symbol) combined with collagen fibers are around the interlobular duct (white solid line). The interlobular duct appears a ductal wall composed with cuboidal epithelium. The typical arrangement of normal architecture of pancreatic acinus (white dotted line) can also be seen in Figure 1A. Due to the strong TPEF signals of cell cytoplasm, but the lack of TPEF signals of nuclei, the morphology of acinar cells (red arrows) with grape-like clusters could be distinguished in this figure as well. The acinar cells appear uniform and maintained their polarity with a distinct nucleus. The non-fluorescent cell nuclei present a round black-hole which is close to the basolateral cell membrane in normal pancreatic tissue (Hu et al., 2012). The same pattern of cellular architecture is also clearly presented in H&E stained images (Fig. 1B), where the positions of pancreatic acinus (black dotted line), the interlobular duct (black solid line), fibrous connective tissue (black triangle symbol), and acinar cells (black arrows) are readily correlated with the corresponding positions in the TPEF imaging.

**TPEF Microscopy Imaging of the Pancreatic SPT**

SPT of pancreas is histologically characterized by solid pseudopapillary areas and cystic spaces (Mahida et al., 2015). The representative large-area TPEF image of the solid pseudopapillary areas of pancreatic SPT is shown in Figure 2A. The cytoplasm of tumor cells can produce strong TPEF signals, while the nuclei are lack of TPEF signals. It allows to easily identify the position of an individual cell. It can be observed from TPEF image that the solid areas are composed of monotonous polygonal epithelioid cells (white arrows) with minimal atypia. These cells are formed by a process of degeneration with cellular disintegration. The nuclei were ovoid and folded with indistinct nucleoli and few mitoses (Liu et al., 2010). The cells are arranged in consistent alignment and accompanied by innumerable capillary-sized vessels (red arrows). These thin-wall blood vessels are too fragile to maintain complete structure, that make it easy to occur hemorrhage and necrosis (Sperti et al., 2008). The typical pseudopapillary structure (white solid line) can also be observed that is made of cells aligned around fine vessels. All these observations are readily correlated with the corresponding standard H&E stained image (Fig. 2B): epithelioid cells (black arrows), capillary-sized vessels (red arrows) and the pseudopapillary structure (black solid line).

Other common histological findings of the pancreatic SPT arranged in cystic spaces are also seen by TPEF image as shown in Figure 3A. As another characteristic of the tumor, hemorrhage that manifests jelly-like or cystic tissue (red arrows) is clearly observed. The evidences of cellular degeneration can also be observed in Figure 3A, including aggregates of foamy histiocytes (white arrows), collection of hyaline stroma (white triangle symbol) and cholesterol crystals (yellow arrows). Based on the cellular and nuclear
pleomorphism in TPEF microscopic images, we could also clearly recognize the epithelial tumor cells existing clusters but discohesive arrangement and often showing cytoplasmic vacuolization in a perinuclear location (Chetty and Serra, 2008). These histopathological features of TPEF microscopic imaging in the cystic spaces were comparable with the corresponding H&E stained image (Fig. 3B), including hemorrhage (red arrows), aggregates of foamy histiocytes (black arrows), collection of hyaline stroma (black triangle symbol), and cholesterol crystals (yellow arrows).

Quantitative Analyses of the Normal Pancreas and Pancreatic SPT Tissues

To characterize the cytomorphological features of the pancreatic SPT different from the normal pancreas, the N/C ratios of normal and tumor cells based on the TPEF and H&E images were calculated and shown in Figure 4. In detail, based on TPEF images, the N/C ratio of normal cells is 0.30 ± 0.06, whereas 0.94 ± 0.12 is for tumor cells. It is obvious that the N/C ratio of tumor cells is significantly greater than that of normal cells (p < 0.001). In addition, based on H&E images, the N/C ratio is 0.42 ± 0.08 in normal tissue, whereas 0.95 ± 0.14 is in the pancreatic SPT tissue (p < 0.001). The quantification result based on TPEF images is similar to that based on H&E images, indicating that TPEF microscopy has the ability to make an optical diagnosis of pancreatic SPT without H&E stains.

Discussion

As there usually present no specific clinical symptoms of SPT and its origin is still unknown, SPT of
pancreas is easy to be misdiagnosed (Yang et al., 2009). It often results in a recurrence postoperation and metastasis (Tajima et al., 2012). Currently, the golden standard for diagnosing the SPT of pancreas is still required, the traditional H&E staining, which is time-consuming and environment-polluting. In this work, we demonstrated that TPEF microscopy with several advantages such as unfixed, rapid, and non-pollution, has the ability to image the histopathological features of pancreatic SPT without the need of extra staining. It clearly visualize the cytoplasm and nucleolus of epithelial cell and collagen in stroma in normal pancreas, as well as the variations such as solid and pseudopapillary structures, foamy histiocytes, hyaline stroma, and cholesterol crystals in SPT of pancreas (Mortenson et al., 2008; Cai et al., 2013). Based on the special pathological morphology, the SPT of the pancreas can be well-differentiated from the normal pancreas and other pancreatic tumors. Moreover, the N/C ratios from TPEF images were extracted to distinguish the pancreatic SPT from normal pancreas. All the results from TPEF images were comparable to their corresponding H&E images. Notably, unstained histological sections avoided the effect on TPEF imaging contrast and resolution due to the excessive or inadequate staining of H&E. Imaging of unstained sections resulted in more accuracy in locating the position of nuclei and nucleolus as well as assessing the area of nuclei and nucleolus, which was of great value in cancer diagnostics. Therefore, TPEF microscopy can present high-contrast and high-resolution images of unstained histological sections at a subcellular level, comparable to the histological results but avoiding the staining problems of H&E. It has great application prospects in the early histological diagnosis with large numbers of samples without H&E staining for SPT of the pancreas in the future.

Conclusion

In conclusion, we presented the microstructures of normal human pancreas and SPT of the pancreas by using the TPEF microscopy without any additional stain, which were consistent with H&E staining images. TPEF microscopy has the ability to achieve the similar pathologic diagnostic information from unstained tissues compared with H&E staining. In the future, TPEF microscopy has the potential to act as an efficient diagnostic tool for non-invasive diagnosis with large numbers of samples of pancreatic tumors.

References

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