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Multiphoton microscopy: A novel diagnostic method for solid tumors in a prospective pediatric oncologic cohort, an experimental study☆

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ABSTRACT

Background: The prognosis of solid pediatric tumors strongly correlates with accurate staging and complete local control. Currently, surgeons rely on macroscopic cues and intraoperative cryosection to determine resection borders. Multiphoton Microscopy (MPM) is a real time technique that allows imaging of tissue without time-consuming tissue processing.

Purpose: This pilot study evaluates the diagnostic potential of MPM in pediatric solid tumors compared to routine histopathology.

Methods: Slides of pediatric tumor samples (nephroblastoma and neuroblastoma [n = 2]; ganglioneuroma, pleuropulmonary blastoma, hepatocellular carcinoma [n = 1]) were prepared to allow direct comparison of MPM with conventional light microscopy. Additionally, we applied MPM to native tumor tissue blocks to evaluate direct visualization of malignant cells through the tumor capsule. Images were interpreted by an attending surgical pathologist. Detectability of tumor-specific features was compared between MPM and conventional histology.

Results: A total of 7 tumors from 7 recruited patients were analyzed. All MPM images were accurate in diagnosing typical criteria of each particular neoplasm. In addition, MPM clearly visualized tumors through the capsule without sectioning or labeling procedures. The quality of MPM was sufficient to make the diagnosis and visualize typical entity-specific architectural changes.

Conclusion: MPM is comparable to conventional histopathology in the diagnosis of pediatric solid tumors without the need for fixation or staining. It therefore has tremendous potential for future real-time intraoperative diagnostics and as an alternative to conventional frozen section histopathology.

Level of evidence: III.

1. Introduction

Exact histopathologic diagnosis is a cornerstone in the successful treatment of pediatric solid tumors, for which complete local control within an uncompromised resection margin dramatically decreases the risk of recurrence. So far, resection margins are usually based on macroscopic features, palpatory feedback, or biopsy. Intraoperative frozen section, however, often prolongs the operation due to time-consuming transport of the sample, fixation, slicing, and staining [1]. Although overall sensitivity of up to 89% has been reported [2], many times the results of frozen section biopsies are inconclusive [3].

Multiphoton Microscopy (MPM) is a relatively novel imaging technique based on non-linear optics and near-infrared femtosecond laser light that possesses specific advantages over other in-vivo techniques [4]. It can provide real-time detailed information about tissue architecture and cell morphology in live tissue using a combination of cell autofluorescence and second harmonic generation. Thus, MPM has the theoretic potential to noninvasively evaluate and analyze morphological structures and the functional states of living tissues. This technique not only offers adequate depth penetration, but also allows in-

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vitro and ex-vivo diagnosis of the unprocessed tissues without labeling or staining [5]. Therefore, MPM may serve as an alternative or adjunctive diagnostic tool in the operative treatment of pediatric solid tumors.

Recently, the feasibility of MPM has been investigated in various entities in adults, including melanoma [6], gastric cancer [7], bladder cancer [8] and rectal cancer [9]. To our knowledge, there has been no report using MPM for pediatric solid tumors. This pilot study therefore explores the potential of MPM in terms of feasibility, utility, and diagnostic accuracy for a variety of childhood neoplasms compared to routine conventional histopathology.

2. Materials and methods

2.1. Study cohort

After registering the study and receiving approval from the ethical review committee of the State (Number 837.274.15), patients were prospectively enrolled from January until December 2016. Informed consent was obtained from all participating caregivers. All patients operated primarily for solid tumors in our department at the state's only university children's hospital qualified for the study. Exclusion criteria included recurrent disease, second-look operation, extensive tumor necrosis that did not allow harvesting of a representative solid tissue block, as well as refusal or inability to provide consent. The case series is reported in compliance with the PROCESS Guidelines.

Native paraffin-embedded tissue blocks were analyzed by MPM. Conventional histopathology was performed on tissue slides from the corresponding tumor region.

2.2. MPM imaging

Imaging was performed using a two-photon laser-scanning microscope (TPLSM, Leica TCS MP5; Leica Microsystems, Wetzlar, Germany). Fig. 1 shows a schematic setup of the microscope. The acquired images were exported as TIFF files and post-processed by Photoshop software (Adobe Systems Software Ltd., Dublin, Ireland).

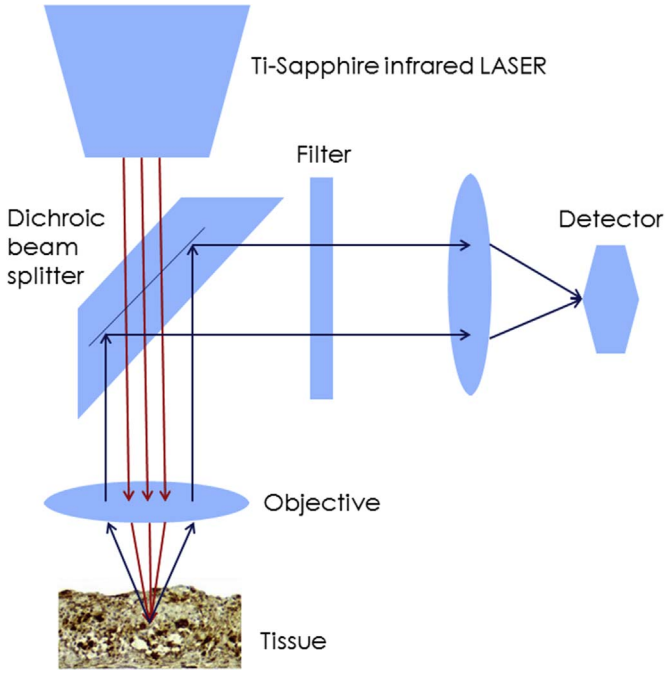

Fig. 1. Schematic setup of the multiphoton microscope. Focal tissue is excited using pulsed laser light (red arrows). The resulting signal is processed through an objective/splitter/filter system and captured by a detector to create the image. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The tissues were excited using a tunable femtosecond pulsed titan-saphire-laser at 950 nm (Chameleon Ultra, Coherent Inc., Santa Clara, CA, USA), controlled by Leica LAS-AF Software (Leica Application Suite, Leica Microsystems, Wetzlar, Germany). Images were obtained through a Leica HCX IRAPO L 25×/0,95 W objective and a BS 505 beam splitter. Two separate filters were used (CFP BP 483/32 nm [cyan], YFP BP 535/30 nm [yellow]).

The CFP BP 483 signal captures autofluorescence of the tissue and was color coded in green, while the YFP BP 535 signal represents second harmonic generation and was color coded in red. Hence, intracellular components are featured in green, while collagen, actin, myosin and tubulin is depicted in red on the final post-processing MPM images.

Field of view was set at 620 μm × 620 μm. Higher scanner zoom was used when necessary. In order to increase the penetration depth within the tissue, the detection unit was placed in immediate vicinity of the sample. Z-stacks of multiple images were produced by collecting a series of images moving from the tissue surface toward deeper layers.

2.3. MPM tumor visualization through the capsule

Paraffin-embedded tissue blocks of the tumor were subsequently imaged by MPM directly through the capsule without further tissue processing. The samples were set up in a petri dish filled with phosphate-buffered saline, and immobilized within a 1 cm diameter stainless steel metal ring to prevent any motion during imaging. Imaging stacks were obtained of a tumor volume approximately 1 mm^3 in dimension and stored electronically in the fashion described above.

2.4. Comparison of images of MPM versus conventional microscopy

For the direct comparison of MPM with conventional histopathology (HP), an average of 16 slides were prepared from the tumor blocks. All slices were cut with a microtome to a thickness of 3 μm to facilitate imaging of the same structures by both methods. The HP slides were processed with conventional hematoxylin-eosin (HE) staining.

2.5. Image analysis and comparison

Images obtained by both MPM and conventional histopathology were independently analyzed by both a board-certified attending pediatric pathologist (LS) and a pediatric surgeon (OM) blinded to the tumor type and results. For each tumor, three representative image pairs (MPM/HP) were shown to the evaluators for interpretation. Typical features of each specific tumor were established beforehand and presence of these features was either confirmed or refuted on MPM and the corresponding conventional HE pathology images in a simple grading system (0 [not visible], + [suggested without details], ++ [recognizable without further details], +++ [recognizable including all details]). Any initial discrepancy between observers was solved by consensus discussion.

3. Results

Tumors included in the study were nephroblastoma (n = 2), neuroblastoma (n = 2), ganglioneuroma (n = 1), pleuropulmonary blastoma type III (n = 1), and fibrolamellar hepatocellular carcinoma (n = 1). Side-to-side comparison of conventional histopathology and corresponding MPM imaging demonstrated excellent definition of tissue characteristics and cellular structures by both methods. No staining of the tissues was required for MPM to generate interpretable images with adequate diagnostic relevance. Typical features and their detectability as graded by the blinded investigators are found in Table 1. In general, HE staining had the advantage of better