April 2017

Scientific Research Process

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 32886
Manuscript Type: CLINICAL TRIAL STUDY

Title: **Characterizing gastrointestinal stromal tumors and evaluating neoadjuvant imatinib by sequencing of EUS-biopsies**

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1 What did this study explore?

This is a prospective, clinical study evaluating and exploring EUS-guided biopsy sampling (EUS-FNB) as the approach for diagnosing and characterizing gastrointestinal stromal tumors at the pretreatment stage. The study analyzed EUS-FNB for the purpose of diagnostic pathology including immunohistochemistry. Furthermore FNB-tissue was evaluated for the purpose of defining the tumor mutation profile regarding *KIT* and *PDGFRA* by Sanger sequencing and the tumor proliferation rate by Ki-67-indexing. Finally, the pretreatment information obtained was compared with equivalent data in surgical specimens to explore the ability to predict and evaluate tumor response to neoadjuvant imatinib.

2 How did the authors perform all experiments?

EUS

Dr Riadh Sadik was the study endosonographer and responsible for the EUS-guided sampling procedures including the FNB-tissue acquisition.

Pathology

Professor Ola Nilsson was the study pathologist and responsible for the assessment of tissue samples including the immunohistochemistry laboratory processing, which was performed according to clinical routine.

Ki-67-indexing

Professor Ola Nilsson was responsible for this part of the study with assistance from dr Per Hedenström.

The K-67-immunostaining of FNB-tissue was performed according to clinical routine. The Ki-67-index of FNB-biopsies was calculated in detail on printouts of digital images captured via an x40-magnification objective (Eclipse E1000, Nikon, Japan) with a ProgResC7-camera (Jenoptik, Germany). Manual counting is of positive nuclei including 2000 tumor cells was performed. Eyeballing and digital counting are considered less accurate and were not used. The result was recorded as the fraction of positive tumor cells (%). Similarly, the Ki-67-index of the respective surgical specimens was analyzed in resected subjects.

Sanger sequencing

Assistant Professor Fredrik Enlund was responsible for this part of the study with assistance from Carola Andersson.

Five µm thick sections from each FNB- and surgical resection sample were cut and pooled into a 1.5 ml tube. DNA was isolated using QIAamp DNA FFPE tissue kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's instructions. 200 ng of DNA was used to detect mutations in *KIT* exon 9, 11, 13 and *PDGFRA* exon 12, 18 with in house designed primers (available on request) and the Multiplex PCR kit (Qiagen) according to the manufacturer's instruction. Sanger sequencing of the amplicons were performed with both the forward and the reverse primers using the BigDye™ Terminator v1.1 Cycle Sequencing Kit with the ABI PRISM™ 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

3 How did the authors process all experimental data?

Dr Per Hedenström was responsible for this part of the study with assistance from Assistant Professor Riadh Sadik.

All data from the experiments and analyses above together with data from the medical files were entered into a study specific SPSS-file. Relevant parameters were classified as categorical or continuous variables. Some variables were dichotomized if that was regarded relevant. No further processing of data was performed. The appropriate statistical test was chosen for each outcome parameter. The 95 % confidence interval was used to present the data as far as possible.

4 How did the authors deal with the pre-study hypothesis?

We hypothesized that the EUS-guided sampling of GIST is more sensitive and accurate if performed with a 22 gauge reverse bevel fine-needle biopsy (EUS-FNB) compared with standard fine-needle aspiration (EUS-FNA). Furthermore, we hypothesized that FNB-tissue is appropriate both for the assessment of the Ki-67-index (as a marker of the tumor proliferation rate) and for the DNA-sequencing of the *KIT*- and *PDGFRA*-genes (as guidance for imatinib therapy).

Dr Per Hedenström, Dr Bengt Nilsson, Dr Ola Nilsson, and Dr Riadh Sadik were responsible for the formulation of the pre-study hypothesis.

Prior to the interventional phase of the study, a sample size calculation was performed for paired, dichotomous variables (statistical power=80%, alpha error=0.05) aiming to detect a difference in sensitivity of 35 % comparing EUS-FNA and EUS-FNB at dual sampling. Available publications of the field and old data from our own institution were used to estimate the baseline sensitivity of EUS-FNA.

We estimated that a minimum of twenty resected patients would be necessary to enable a comparison of the FNB-tissue with the surgical specimen tissue with respect to the Ki-67-index and the mutational status of *KIT* and *PDGFRA*. We considered a complete mutation match (FNB-tissue *vs* surgical specimen) in a minimum of 80 % of the cases as adequate.

5 What are the novel findings of this study?

- EUS-FNB is safe, highly accurate, and more appropriate than EUS-FNA for the purpose of the preoperative diagnosis of gastrointestinal stromal tumors.
- FNB-tissue is accurate for clarifying the individual tumor mutation profile regarding *KIT* and *PDGFRA* at the preoperative stage
- In patients not treated with imatinib prior to surgical resection, the tumor proliferation rate, measured as the Ki-67-index, in FNB-tissue is comparable with that assessed in surgical specimen tissue
- In patients treated with imatinib prior to surgical resection, the tumor proliferation rate, measured as the Ki-67-index, in FNB-tissue is higher and probably more reliable than that assessed in surgical specimen tissue
- This extensive, diagnostic information obtained at the preoperative stage opens up for an early, personalized treatment of patients with GIST

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*

Sincerely Yours

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