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The Utility of Intraoperative Cytological Smear and Frozen Section in the Surgical Management of Patients with Cushing's Disease due to Pituitary Microadenomas

Nidan Qiao[1,2] · Brooke Swearingen[2,3] · E. Tessa Hedley-Whyte[2,4] · Nicholas A. Tritos[2,5]

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Abstract

Cushing's disease (CD) is most commonly caused by a microadenoma, which at surgical exploration may not provide adequate tissue for pathologic diagnosis using standard techniques. We wished to determine the accuracy of intraoperative pathologic examination and whether the addition of intraoperative cytology increased the diagnostic yield. We reviewed the pathology reports from 403 operations on 341 patients with CD microadenomas from a single institution. The concordance rates of intraoperative diagnoses (cytology and frozen) with the final (paraffin section) pathological diagnosis were calculated. The overall pathologic confirmation of an adenoma (by either cytology, frozen, or paraffin section) was compared with the result from a historical cohort (using only standard frozen section analysis but not intraoperative cytology) and the pooled result from a meta-analysis of previously published data. The concordance rate between frozen section diagnosis and paraffin section histology was 390/403 (96.8%). The concordance rate between cytological smear and paraffin section histology was 213/246 (86.6%). In 54 cases (13.4%) with ultimate remission, pathologic confirmation was obtained only on intraoperative pathology (frozen section or cytology). Overall, pathologic confirmation was obtained in 326 operations (80.9%) by at least one pathological modality. The overall pathological confirmation of an adenoma was greater after the introduction of intraoperative cytology when compared with the historical control (67.1%, $p = 0.015$), and compared with the pooled rate of published data from the meta-analysis (72.1%, $p < 0.001$). Our findings suggest that addition of intraoperative cytological analyses during surgery for CD is an additional useful diagnostic tool for both neurosurgeons and pathologists.

Keywords Diagnosis · Smear · Pathology · Remission · Relapse

Introduction

Cushing's disease (CD) is characterized by hypercortisolism resulting from a corticotroph pituitary adenoma that secretes excess adrenocorticotrophic hormone (ACTH). Macroadenomas (more than 10 mm in greatest diameter on magnetic resonance imaging [MRI]) comprise only a small proportion (about 10%) of tumors in patients with CD [1]; the majority of these tumors are microadenomas. Microadenomas are often difficult to identify on MRIs and can be classified as either MRI-unequivocal (evidence of clear-cut microadenoma on imaging) or MRI-equivocal (without evident microadenoma on MRI).

Transsphenoidal surgery remains the most effective way to treat patients with CD. Postoperative remission rates vary from 61 to 95% across studies [2, 3] but are lower for patients with MRI-equivocal tumors. Risk factors for persistent hypercortisolism after surgical treatment of CD include lack of preoperative and intraoperative tumor detection [4, 5].

✉ Nidan Qiao
nidan_qiao@hms.harvard.edu

✉ Nicholas A. Tritos
ntritos@mgh.harvard.edu

[1] Department of Neurosurgery, Shanghai Institute of Neurological Surgery, Huashan Hospital, Shanghai Medical School, Fudan University, 12 Wulumuqi Zhong Road, Shanghai, China

[2] Harvard Medical School, 25 Shattuck Street, Boston, MA 02115, USAx

[3] Department of Neurosurgery, Massachusetts General Hospital, Boston, MA, USA

[4] C.S. Kubik Laboratory for Neuropathology, Department of Pathology, Massachusetts General Hospital, Boston, MA, USA

[5] Neuroendocrine Unit, Massachusetts General Hospital, 100 Blossom Street, Suite 140, Boston, MA 02114, USA

Though paraffin section histology with immunochemistry has been the gold standard in the pathological diagnosis of ACTH-secreting tumors, up to 35% of tumors are not confirmed with routine pathological sections [5–9].

Pathological identification of an adenoma is important to confirm the pituitary origin of the hypercortisolism, even after an extensive preoperative diagnostic evaluation. It may not be obvious whether failure to achieve remission after an apparently negative exploration because a pituitary tumor, though present, was not found, or whether the presumed diagnosis of CD was in error. Pathological confirmation of CD is also useful in guiding further therapy, especially after unsuccessful exploration (e.g., in justifying the need for postoperative radiosurgery). In addition, tumor tissue is useful for investigations into the molecular pathogenesis of these tumors.

The preservation of tissue architecture after paraffin embedding is optimal for pathologic diagnosis, and also allows immunohistochemical confirmation. In some cases of poorly defined microadenomas, however, the minute quantities of tissue available for processing preclude a definitive paraffin section tissue diagnosis [10]. In an attempt to preserve all available tissue for pathologic confirmation, cytological smears and/or touch preps have been prepared by the surgeon at the operative field and sent to the pathology laboratory for intraoperative evaluation since 1999 (Fig. 1). If an adenoma was identified, further specimens from the presumed tumor bed were later sent for frozen section analysis to identify residual tumor and confirm negative margins, followed up by paraffin histology if enough tissue was available. In patients with a negative cytological smear and negative follow-up intraoperative specimens, a hemi-hypophysectomy was performed, guided by the lateralization of the inferior petrosal sinus sampling (IPSS).

Cytologic smears have been reported to be highly accurate in the intraoperative diagnosis of pituitary adenomas [11–13], but the utility of intraoperative cytology in CD, where the tumors are often hardest to detect, is not well-described. There is a single report of the use of cytological analysis in ACTH adenomas [14]. It is also uncertain whether the addition of intraoperative cytology improves the diagnostic yield of pathologic diagnosis in CD or correlates with clinical outcomes.

We hypothesized that both cytology and frozen section analyses during surgical exploration for CD are highly accurate. Furthermore, we hypothesized that adding the cytologic smears prepared by the neurosurgeon on the operative field to routine frozen and paraffin section pathology might increase the rate of pathologic confirmation of microadenomas, and perhaps improve the rate of postoperative remissions. To investigate whether intraoperative pathology has diagnostic or prognostic value, we retrospectively analyzed the case records of patients with CD attributed to a microadenoma who underwent transsphenoidal pituitary surgery in our institution between 1999 and 2017.

Methods

Patient Population

We reviewed available records of all patients with CD attributed to a microadenoma who underwent transsphenoidal surgery between 1999 and 2017. Patients with macroadenomas were excluded. All microadenomas were smaller than 1 cm in greatest diameter. Microadenomas were classified as either MRI-unequivocal (evidence of clear-cut microadenoma on imaging) and MRI-equivocal (without evident microadenoma on MRI) tumors. One neurosurgeon (BS) performed all the procedures. The study was approved by the hospital Human Research Committee (Institutional Review Board) and was conducted under the ethical standards of the Declaration of Helsinki. The Institutional Review Board waived the

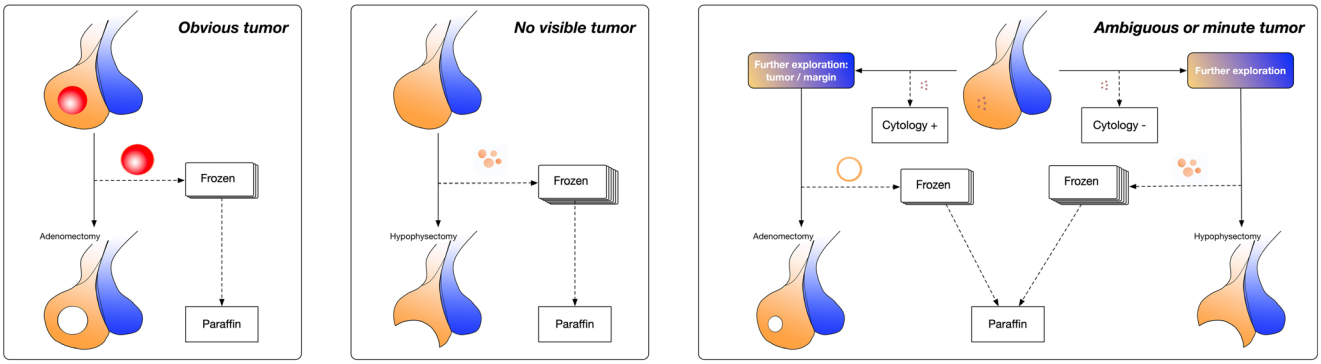

Fig. 1 Surgical (solid line) and pathological (dotted line) workflow for patients undergoing surgical exploration for Cushing's disease. Adenomectomy was performed in patients with obvious tumor, with/without margin sent for frozen section analysis. For those patients whose specimens were ambiguous or minute, cytological smears were sent for rapid evaluation. If an adenoma was identified, further specimens from the presumed tumor bed were later sent for frozen section analysis to identify residual tissue and confirm negative margins. In patients with a negative cytological smear and negative follow-up specimens, a hemi-hypophysectomy was performed, guided by the lateralization of the inferior petrosal sinus sampling (IPSS). All frozen tissue remnants were submitted for paraffin processing

requirement for patient consent for this retrospective study which did not involve direct patient contact.

Criteria for Diagnosis of CD and Clinical Outcomes

The diagnosis of Cushing's syndrome was based on at least two of the following criteria: 1) elevated 24-h urinary free cortisol (UFC) above the upper limit of the reference range; 2) elevated late-night salivary free cortisol (LNSC), and/or 3) lack of morning serum cortisol suppression after 1 mg dexamethasone in the absence of other exogenous glucocorticoid use. In patients with Cushing's syndrome, CD was confirmed by normal or elevated preoperative plasma ACTH concentrations and at least one of the following: 1) preoperative bilateral inferior petrosal sinus sampling (IPSS) predicting a pituitary source; 2) histologic diagnosis of adenoma including ACTH immunohistochemistry; 3) clinical and endocrine remission after pituitary surgery [13].

Postoperative remission was defined as the postoperative presence of low early morning serum cortisol concentrations (< 5 mcg/dl), or low 24-h UFC levels (< 20 mcg/24 h) on multiple specimens. Recurrence was defined as re-presentation with an elevated 24-h UFC, inability to suppress serum cortisol after low-dose dexamethasone testing, or elevated LNSC with clinical symptoms after initial remission [15].

Pathological Confirmation

Intraoperative Cytology Tissue retrieved by the transsphenoidal curette was transferred onto a dry sterile microscope slide as either a touch prep or a squash prep using a second slide to "smear" the tissue across the first slide. The slide was immediately fixed in 95% ethanol and submitted to the pathology laboratory for hematoxylin and eosin (H&E) staining and analysis (preparation time approximately 2–4 min). The diagnosis of adenoma was based on the presence of a uniform population of cells with uniform nuclear to cytoplasmic ratio (Fig. 2(A)) as opposed to anterior pituitary smears which are composed of cells of varying sizes and varying nuclear-cytoplasmic ratios (Fig. 2(B)). We had earlier used smears from post mortem anterior pituitaries as controls.

Intraoperative Frozen Section A sample of the tissue was sent to the pathology laboratory for rapid diagnosis. Routinely, a tiny portion of the fresh tissue sample was "smeared," and the smear was fixed in alcohol and stained with H&E. The remainder was mounted in Tissue-Plus™ O.C.T. compound (Fisher Healthcare, Norwich, USA) and frozen in liquid nitrogen and 5-micron sections were stained with toluidine blue and/or H&E. The frozen remnants were thawed in formalin for subsequent paraffin processing. Paraffin sections were stained with H&E and immunostained for ACTH and other hormones. The diagnosis of the cytological smear preceded all other diagnostic histology and was the only pathological analysis in cases where no further tumor was obtained for diagnostic analysis. All slides were reviewed by neuropathologists with experience in the diagnosis of pituitary adenomas.

The pathology reports were reviewed for the presence (positive) or absence (negative) of adenoma in intraoperative cytology, frozen section or paraffin section diagnoses.

Concordance and Discordance

We calculated the concordance rate between the intraoperative pathologic diagnosis and final pathologic confirmation. A case was considered concordant when positive intraoperative pathology was followed by either subsequent paraffin confirmation or postoperative remission, or when intraoperative pathology was negative and subsequent paraffin sections were also negative. All other cases were considered as discordant.

The Control Groups

We used data from operations performed in our institution before the introduction of intraoperative cytology in 1999 (from 1990) as a historical control. The overall pathological confirmation rate (confirmation of an adenoma in either one of pathological modalities) was compared between the current cohort and the historical cohort.

We further used the pooled published data on pathological confirmation rates from a meta-analysis as an external control. To identify the pertinent articles, we searched PubMed (using the terms "Pituitary ACTH Hypersecretion/surgery"[Mesh] or

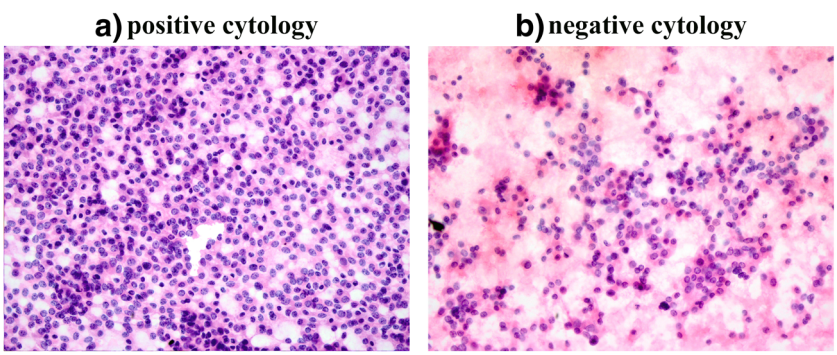

Fig. 2 Smears prepared by the neurosurgeon. A is from a case positive for adenoma. Note the uniform small cells with round nuclei and moderate amounts of cytoplasm. B is an example of smears considered "negative for adenoma." Note that the relatively sparse cells vary in size and in relative amounts of cytoplasm. Hematoxylin and eosin staining. Original magnifications × 400

"CD surgery") for any reported pathological confirmation rate in surgical series of CD from microadenomas. We excluded studies published before 1999, studies focusing on childhood-onset CD, and studies with combined analysis of macroadenomas and microadenomas.

Statistical Analysis

Continuous variables (including age) were described as mean and standard deviation. Categorical variables (including gender, MRI characteristics, pathologic confirmation rate, endocrine remission) were described as counts and proportions. Relapse was analyzed using a survival (Kaplan-Meier) model. Rates of concordance and discordance between intraoperative pathology and paraffin section histology were calculated. We used the chi-square tests and regression discontinuity design for the comparison with the historical control, and the one-sample proportion test for the comparison with the external control (pooled pathological confirmation rate from the random effects model analysis). All statistical analyses were completed with R software version 3.4.2 (R Foundation for Statistical Computing, Vienna, Austria).

Results

We identified 341 patients (286 females, age 42.2 ± 14.4 years) fulfilling the criteria for CD due to a microadenoma. In total, 403 operations were performed, including 302 initial operations, 25 early second operations, and 76 operations after recurrence (Table 1).

Diagnostic Utility of Frozen and Cytological Pathology

Intraoperative frozen sections were performed in all cases (Table 1), 105 operations with an obvious tumor, 52 operations with no visible tumor at surgery, and 246 operations with an ambiguous or minute tumor. Two hundred eighty-nine operations had a frozen section diagnosis of adenoma with confirmation by subsequent paraffin sections or post-surgical remission. One hundred one operations yielded both a negative frozen section and paraffin diagnoses. Seven operations had a positive frozen section which was not confirmed by paraffin sections nor by post-surgical remission. Six operations had a negative frozen section but a positive paraffin diagnosis. Thus, the concordance rate between frozen section and paraffin section histology was 390/403(96.8%), and the discordance rate was 13/403(3.2%).

Intraoperative cytology was performed in 246 operations (Table 2). In 172 of these operations, positive cytology (Fig. 2A) was accompanied by a subsequent positive paraffin diagnosis or post-surgical remission. Forty-one operations had both negative cytological (Fig. 2B) and paraffin results. Positive cytology without paraffin confirmation in the subsequent samples or post-surgical remission occurred in 11 operations. Cytology was negative in 22 procedures with positive paraffin sections in subsequent samples. Among these 22 cases, 18 cytological diagnoses were attributed to unrepresentative sampling. Other diagnoses included cohesive cells (2), possible Crooke's change (1), and basophil hyperplasia (1). Thus, the concordance rate between cytology smear and paraffin section histology was 213/246(86.6%), and the discordance rate was 33/246(13.4%).

The concordance rate of cytology smears was also similar between MRI-equivocal tumors (90/109, 82.6%) and MRI-unequivocal tumors (123/137, 89.8%, $p = 0.144$). The concordance rate of frozen section was also similar between MRI-equivocal tumors (170/177, 96.0%) and MRI-unequivocal tumors (220/226, 97.3%, $p = 0.653$).

Additional Diagnostic Yield with Cytopathology

In the whole cohort, 54 operations (13.4%) with positive intraoperative diagnoses (either frozen or cytology section) without paraffin confirmation had remission, including 17 operations diagnosed only by frozen section, 16 operations diagnosed only by cytology, and 21 operations confirmed by both cytology and frozen section.

Overall Rate of Pathologic Confirmation

The overall rate of pathological confirmation as defined by the presence of an adenoma on either cytology, frozen, or paraffin sections was 326/403(80.9%).

Historical and External Comparisons

With the introduction of intraoperative cytology in 1999, the overall rate of pathologic confirmation of an adenoma increased from 47/70 (67.1%) between 1990 and 1998 to 80.9% after 1999 ($p = 0.015$, Table 3). In the regression discontinuity analysis, assuming other confounding factors did not change abruptly at the threshold point (early 1999), we observed discontinuity of the overall pathological confirmation rate before and after the introduction of intraoperative cytology (Fig. 3).

We identified 14 published studies that reported pathological information in CD in our meta-analysis [6–9, 16–25] (Fig. 4A). The pooled data indicate that pathological confirmation was obtained in 72.2% (95%CI, 66.1–77.5%) of microadenomas. By comparison, the approach outlined herein yielded a higher overall rate of pathological confirmation (80.9%, $p < 0.001$). Accounting for the proportion of MRI-unequivocal tumors as a possible confounding factor, the meta-regression analysis showed that our pathological

Table 1 Total operations stratified by intraoperative findings

	Total operations (403)	Subgroup by intraoperative findings		
		Obvious tumor (105)	No visible tumor (52)	Ambiguous or minute tumor (246)
Surgical subtypes				
Initial	302(74.9%)	88(83.8%)	29(55.8%)	185(75.2%)
Relapse	76(18.9%)	13(12.3%)	16(30.8%)	47(19.1%)
Early second	25(6.2%)	4(3.8%)	7(13.5%)	14(5.5%)
Frozen confirmation	296(73.4%)	105(100.0%)	11(21.2%)	180(73.2%)
Paraffin confirmation	257(63.8%)	97(92.3%)	3(5.8%)	157(63.8%)
Number of frozen specimens	2.7 ± 0.2	2.2 ± 0.1	4.5 ± 0.3	2.5 ± 0.2
Remission	320(79.4%)	97(92.3%)	30(57.7%)	193(78.5%)
Diagnostic utility of frozen section				
Concordance	390(96.8%)	104(99.0%)	49(94.2%)	237(96.4%)
Discordance	13(3.2%)	1(1.0%)	3(5.8%)	9(3.6%)

confirmation rate was higher than the confidence interval of the regression line (Fig. 4B).

In the entire cohort, remission was achieved in 320 operations (79.4%). The 5-year recurrence-free survival rate after the initial surgery was 83.0% (95%CI, 77.1–89.3%). Both outcomes were similar compared with our historical cohort and meta-analysis.

Discussion

In this study, we report on the diagnostic and prognostic values of intraoperative frozen section and cytological diagnosis in patients with CD undergoing transsphenoidal surgery. Previous studies have indicated that a risk factor for persistent hypercortisolism after surgical treatment of CD is the failure to detect tumor intraoperatively [4, 5]. Although the use of intraoperative frozen section and cytological smear has been reported in patients with pituitary adenomas, no studies have specifically addressed whether intraoperative cytology adds to the pathological detection of tumor and surgical outcome in patients with CD due to equivocal microadenomas.

Intraoperative frozen sections can be beneficial to the surgeon by confirming the presence of abnormal tumor tissue, evaluating the adequacy of resection, and limiting unnecessary dissection of the pituitary gland with its attendant risk of pituitary insufficiency. Several studies have suggested that multiple biopsies with frozen sections may be needed for accurate localization and complete resection of ACTH-secreting tumors [26, 27]. The accuracy of frozen section pathology in this study is comparable with that reported in previous studies, which ranges from 83.1 to 94.3% [26–30].

Cytology from biopsies submitted to a pathologist has been shown to be highly accurate (ranging from 79.0 to 94.9%) in the diagnosis of central nervous system tumors, including pituitary adenomas [11, 12, 31]. Our results on intraoperative cytology, prepared by the surgeon, support and extend these findings. It has previously been reported that PAS staining and

Table 2 Operations with intraoperative cytology stratified by surgical subtypes

	Total operations with intraoperative cytology (246)	Subgroup by the surgical subtypes		
		Initial (185)	Relapse (47)	Early second (14)
Cytological confirmation	183(74.4%)	141(76.2%)	32(68.1%)	10(71.4%)
Frozen confirmation	180(73.2%)	142(76.8%)	29(61.7%)	9(64.3%)
Paraffin confirmation	157(63.8%)	123(66.5%)	26(55.3%)	8(57.1%)
Number of frozen specimens	2.5 ± 0.2	2.5 ± 0.1	2.1 ± 0.2	3.1 ± 0.5
Remission	193(78.5%)	151(81.6%)	33(70.2%)	9(64.3%)
Diagnostic utility of cytology				
Concordance	213(86.6%)	160(86.5%)	45(95.7%)	8(57.1%)
Discordance	33(13.4%)	25(13.5%)	2(4.3%)	6(42.9%)

Table 3 Operations before and after the introduction of intraoperative cytology

	Before 1999 (70)	After 1999 (403)	*p* value
Surgical subtypes			0.371
Initial	53(74.9%)	302(75.7%)	
Relapse	10(18.9%)	76(14.3%)	
Early second	7(6.2%)	25(10.0%)	
Frozen confirmation	45(64.3%)	296(73.4%)	0.139
Paraffin confirmation	43(61.4%)	257(63.8%)	0.809
Cytological confirmation	NA	183/246(74.4%)	NA
Overall confirmation*	47(67.1%)	326(80.9%)	0.015
Remission rate	51(72.9%)	320(79.4%)	0.287

NA not available; *overall confirmation was defined by the presence of an adenoma on either cytology, frozen, or paraffin sections

immunohistochemistry on cytology touch preparations are helpful in the diagnosis of ACTH-secreting tumors [32]. However, in this study, we only analyzed the single smears from the operating room and had no other tissue at that time. Attempts at destaining and subsequent immunostaining for ACTH and prolactin (as a control for the immunochemistry) were unreliable. We also found that cytology may not be as reliable in early second surgeries with only 57% accuracy, which may be due to structural disruption as a consequence of previous surgical exploration. The high accuracy of cytology in surgical exploration after recurrence may be due to selection bias (only patients with definite diagnosis were submitted to surgery). Most of the false negative cytology was presumably due to non-representative initial sampling as further exploration with frozen diagnosis identified tumors in 20 out of 22 cases. In false positive cytology cases, samples from a cluster of nodular hyperplasia could resemble the monomorphic population of cells seen in a tumor.

It has previously been reported that adenomatous tissue may be seen only on intraoperative cytology, and not subsequent histological sections, especially in adenomas less than 3 mm [14]. In our study, 13.4% of cases with positive intraoperative pathology lacked tissue for paraffin section analysis, but these cases nonetheless achieved remission suggesting that this approach potentially increases the diagnostic yield of adenoma in CD. While it is conceivable that pathologic confirmation would have been obtained after paraffin sectioning had this tissue survived conventional processing, the minute quantity of tissue available for pathologic analysis in many of these cases was optimally handled using cytopathology techniques.

In our historical comparison, we showed that the rate of pathological confirmation improved after the introduction of cytopathology in our institution. In the external comparison, we investigated the difference between pathologic confirmation rates in CD in our institution, obtained after the introduction of both intraoperative cytology and frozen section pathology, and previously published data on pathological confirmation rates.

There are several limitations to this study.

1. This is a retrospective case series using a historical and literature meta-analysis to validate our approach. The utility of historical controls may be confounded by the additional experience gained by the surgeon in identifying tumor tissue during the period of this study. This may tend to over-estimate the benefit of cytologic examination.
2. Potential selection bias in cytology cases: the decision to perform intraoperative cytology was at the discretion of the surgeon but was made in only those cases where significant diagnostic uncertainty was anticipated. These may be the cases where cytologic examination is most difficult and may tend to under-estimate its accuracy.
3. The use of intraoperative cytology and frozen section pathology may preclude formal histological analysis if there is no additional tissue available.
4. In false positive cases, the surgeon may be dissuaded from performing further exploration which might lead to a lower probability of endocrine remission.

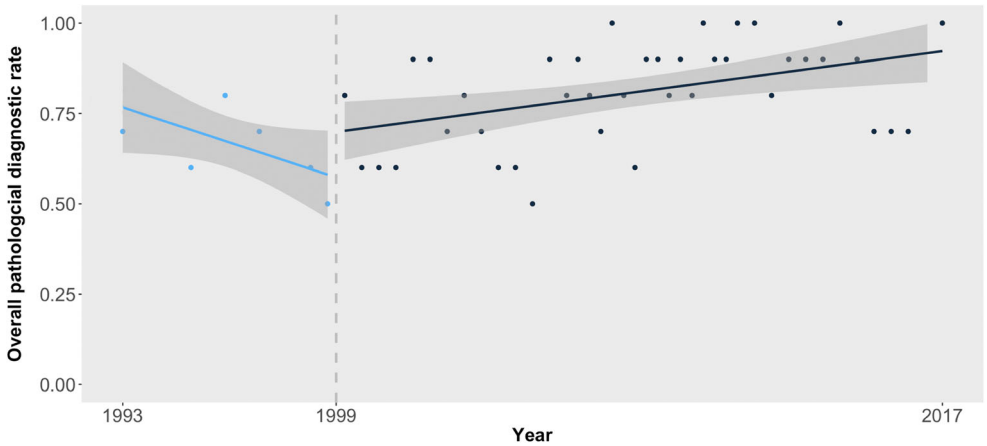

Fig. 3 Regression discontinuity assuming other factors kept constant during the short period at the cut-off point (1999). Overall pathological confirmation rates (*y*-axis) were regressed on the sequence (*x*-axis) before and after the cut-off point, respectively. Every dot represents ten operations. The shaded area represents the 95% confidence interval of the regression line

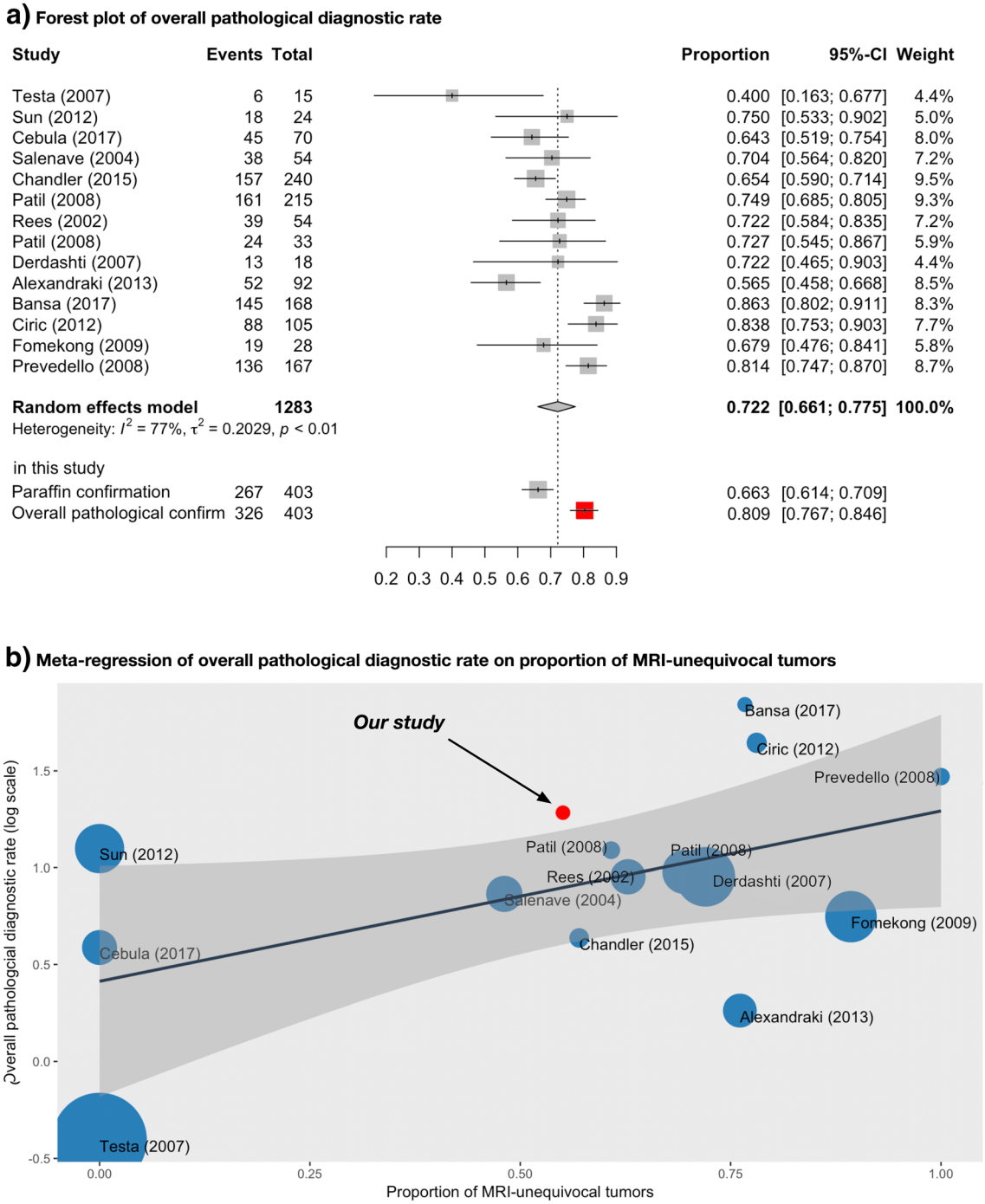

Fig. 4 Comparison of our results to the pooled rate of meta-analysis. A. Forest plot of the meta-analysis of previous studies on overall pathological confirmation in CD diagnosis. Studies were ordered by the proportion of MRI-unequivocal tumors, from 0 to 100%. Events: the presence of adenomas. Total: patients/cases with a microadenoma included in each study. 95% confidence intervals of pathological confirmation in our study are higher than the corresponding pooled rate. B. Meta-regression of overall pathological confirmation (*y*-axis, log scale) on the proportion of MRI-unequivocal tumors (*x*-axis). Every circle represents one study, and the circle size is proportional to the standard error. The solid line represents the regression line with 95% confidence interval band. Our overall pathological confirmation is above the confidence interval band

5. Both frozen section pathology and cytologic interpretation require experienced pathologists, which may impede the generalizability of these techniques to other institutions.

Conclusions

Both intraoperative cytology and frozen section pathology, obtained during surgical exploration for CD, are accurate in

the pathologic confirmation of tumor tissue. The overall rate of pathological confirmation of the diagnosis of CD due to a microadenoma appears to improve by combining intraoperative cytology with frozen section and paraffin pathology.

Compliance with Ethical Standards

The study was approved by the hospital Human Research Committee (Institutional Review Board) and was conducted under the ethical standards of the Declaration of Helsinki.

Conflict of Interest The authors declare that they have no conflict of interest.

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