Immunohistochemical Nuclear Expression of β-Catenin as a Surrogate of *CTNNB1* Exon 3 Mutation in Endometrial Cancer

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ABSTRACT

Objectives: *CTNNB1 exon 3 mutations have shown independent prognostic value in endometrial cancer. We aimed to assess whether nuclear β-catenin expression is an accurate surrogate, as immunohistochemistry is cheaper, faster, and more widely applicable than sequencing.*

Methods: *A systematic review was performed by searching electronic databases for all studies assessing the association between β-catenin immunohistochemical expression and CTNNB1 mutations. Meta-analysis of diagnostic accuracy was performed by calculating sensitivity, specificity, positive and negative likelihood ratios (LR+ and LR–), diagnostic odds ratio (DOR), and area under the curve (AUC) on summary receiver operating characteristic curves.*

Results: *Fifteen observational studies with 1,158 endometrial carcinomas were included. Pooled estimates showed sensitivity = 0.88, specificity = 0.85, LR+ = 4.57, LR– = 0.20, DOR = 27.16, and high diagnostic accuracy (AUC = 0.91).*

Conclusions: *Nuclear expression of β-catenin is an accurate immunohistochemical surrogate of CTNNB1 exon 3 mutations and thus might be considered in the risk stratification of endometrial cancer.*

Endometrial cancer (EC) is the most common gynecologic malignancy in the Western world, with an increase in both incidence and mortality rates in the past years.[1]

Most patients at a low stage have good outcomes and are eligible for conservative treatment in many cases, but patients with advanced stages or more aggressive tumor histotypes show poor prognosis.[2,3] The major cause of increased mortality is a risk stratification that is still based on poor reproducible histologic examination, which has led to over- and undertreatment of patients.[4,5]

In the 2013, The Cancer Genome Atlas (TCGA) identified four distinct molecular categories of EC that correlated with the prognosis. The first category included endometrioid ECs with a very high mutational rate ("ultra-mutated group"); ECs in this group harbored mutations in the polymerase-ε (POLE) exonuclease domain and showed good prognosis. The second category was constituted by microsatellite-instable endometrioid EC with a varying grade, a high mutational rate ("hyper-mutated group"), and an intermediate prognosis. The third category included low-grade endometrioid ECs with a low mutational rate and a low somatic copy number alterations rate ("copy number–low group"), with good to intermediate prognosis. The fourth category showed a low mutational rate but a high somatic copy number alterations rate ("copy number–high group") and *TP53* mutations; this group was mainly constituted by serous EC and showed poor prognosis.[2,6-8]

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Subsequent studies have refined the molecular/prognostic classification, demonstrating that high expression of L1CAM, lymphovascular space invasion, and *CTNNB1* (β-catenin–encoding gene) exon 3 mutations have an independent prognostic value.[9-11] All these findings have pointed out that the traditional systems for risk stratification of EC cause many patients to be over- or undertreated.[2,7,8] Therefore, a molecular definition of EC specimens appears necessary to choose an adequate treatment. Unfortunately, techniques used to identify genomic subgroups, including genome sequencing, have been expensive, complex, and unsuitable for wide clinical application.[2]

This has led to an increasing interest in immunohistochemical surrogates of molecular prognostic markers.[8] Immunohistochemistry is cheaper, faster, and more widely available than sequencing analyses.[12-16] In fact, abnormal immunohistochemical expression of p53 protein and of mismatch-repair proteins seems to be a reliable surrogate for *TP53* mutations and microsatellite instability, respectively.[2,7,8]

Regarding *CTNNB1*, nuclear accumulation of β-catenin has been proposed as an immunohistochemical surrogate of exon 3 mutations, but the accuracy of such a method is not well defined.

The objective of our study was to assess the diagnostic accuracy of β-catenin nuclear accumulation as an immunohistochemical surrogate for *CTNNB1* exon 3 mutations. We aimed to define if immunohistochemistry for β-catenin may be introduced as a reliable test for the prognostic stratification of EC.

Materials and Methods

Methods for electronic search, study selection, risk of bias assessment, extraction, and analysis of data were defined a priori. Two authors (A.T. and A.R.) independently performed all review stages. Disagreements were resolved by discussion with a third author (G.S.). This study was reported following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement[17] and the Synthesizing Evidence from Diagnostic Accuracy Tests (SEDATE) guidelines.[18]

MEDLINE, Scopus, EMBASE, Web of Sciences, Cochrane Library, ClinicalTrial.gov, and Google Scholar were searched from their inception to September 2018 by using a combination of the following text words and all their synonyms found on Medical Subject Headings vocabulary: *beta-catenin*, *β-catenin*, *CTNNB1*, *exon 3*, *endometrial cancer*, *endometrioid adenocarcinoma*, *endometrium*, *immunohistochemical*, *immunohistochemistry*, *prognosis*, *marker*, *Atlas*, *cancer*, *genome*, *TCGA*, *PORTEC*, *ProMisE*, *Proactive Molecular Risk Classifier*, and *TransPORTEC*. Relevant references from each selected study were also assessed.

All peer-reviewed, prospective, or retrospective studies assessing the association between β-catenin immunohistochemical expression and *CTNNB1* mutations were included in the systematic review. Studies not allowing comparisons between immunohistochemistry and molecular analysis were excluded. In case of overlapping data between two studies (ie, same institution and period of enrollment, same immunohistochemical and molecular results), the study assessing the smaller patient series was excluded.

The risk of bias among studies was assessed according to the revised Quality Assessment of Diagnostic Accuracy Studies 2.[19] For each study, four domains related to the risk of bias were assessed: (1) patient selection (ie, if the patients were consecutive or randomly selected from a consecutive series), (2) index test (ie, if the criteria used to assess β-catenin expression were clearly defined), (3) reference standard (ie, if the methods for molecular analysis were appropriate), and (4) flow and timing (ie, if patients were not inappropriately excluded from the index test or reference standard). Reviewers' judgments were "low risk," "unclear risk," or "high risk of bias" for each domain.

Concerns about applicability were also evaluated for domains 1, 2 and 3 (ie, if the criteria used are correct but do not fit the objective of our review).

Original data were not modified during extraction. Two-by-two contingency tables were prepared for each study. Two qualitative variables were reported in the tables. The first variable (which was the index test) was β-catenin expression, dichotomized as "nuclear" vs "membranous/cytoplasmic." The second variable (which was the reference standard) was *CTNNB1* mutational status, dichotomized as "mutated (mt)" vs "wild type (wt)."

For the index test, any striking nuclear β-catenin staining was considered "nuclear." Weak nuclear expression—defined as a light brown nuclear nuance—was lumped together with "membranous/cytoplasmic," as it may occur in normal endometrium.[20] For the reference test, only mutations in exon 3 of the *CTNNB1* gene were considered.

CTNNB1-mt cancers with nuclear β-catenin were considered as true positive, *CTNNB1*-wt cancers with membranous/cytoplasmic β-catenin as true negative, *CTNNB1*-wt cancers with nuclear β-catenin as false positive, and *CTNNB1*-mt cancers with membranous/cytoplasmic β-catenin as false positive.

Sensitivity, specificity, positive and negative likelihood ratios, and diagnostic odds ratio (DOR) were

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calculated for each study and as a pooled estimate. Values were reported graphically on forest plots with 95% confidence intervals (CIs).

Statistical heterogeneity among the included studies was evaluated using the Higgins I^2 statistic; heterogeneity was categorized as null for $I^2 = 0\%$, minimal for $0\% < I^2 \leq 25\%$, low for $25\% < I^2 \leq 50\%$, moderate for $50\% < I^2 \leq 75\%$, and high for $I^2 > 75\%$.

The random-effect model of DerSimonian and Laird was used independently from the heterogeneity, as recommended by the SEDATE guidelines.[18]

Area under the curve (AUC) was calculated on summary receiver operating characteristic curves. The diagnostic usefulness was categorized as absent for AUC ≤ 0.5, low for 0.5 < AUC ≤ 0.75, moderate for 0.75 < AUC ≤ 0.9, high for 0.9 < AUC < 0.97, and very high for AUC ≥ 0.97.

The data analysis was performed using Meta-DiSc version 1.4 (Clinical Biostatistics Unit, Ramon y Cajal Hospital) and Review Manager 5.3 (The Nordic Cochrane Centre, Cochrane Collaboration, 2014).

Results

Fifteen observational studies with 1,158 patients were included. The process of study selection is reported in Figure 1.

Most ECs (82%) were endometrioid adenocarcinoma. Histologic specimens were obtained by hysterectomy in 13 studies and by biopsy in one study, while in the other one, the sampling method was unspecified. DNA or RNA was obtained from fresh-frozen tissue in two studies, from paraffin-embedded tissue in 10 studies, and from either the former or the latter in two studies. Molecular analysis included polymerase chain reaction and exon 3 sequencing in all studies; in one study, next-generation sequencing (NGS) was performed. Single-strand conformation polymorphism was performed as a screening test in five studies.

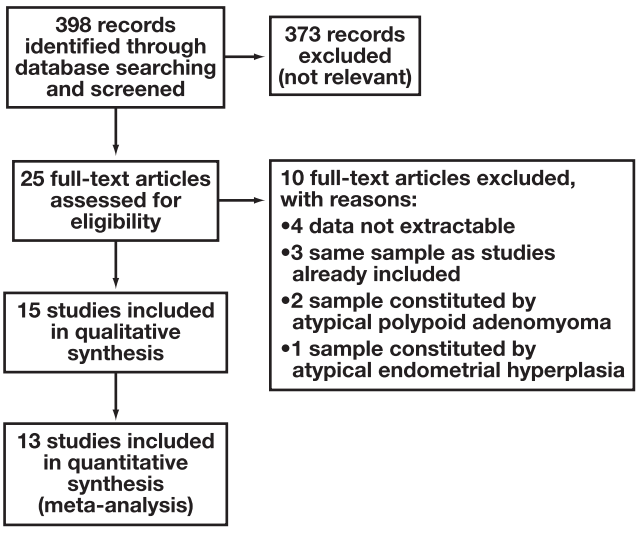

Figure 1 Flow diagram of studies identified in the systematic review (Preferred Reporting Items for Systematic Reviews and Meta-Analyses template).

Characteristics of the included studies are reported in Table 1.

For the "patient selection" domain, all studies were considered at a low risk of bias. Concerns were considered unclear for seven studies (selection restricted to EC with synchronous ovarian cancer,[24,33] EC with coexisting endometrial hyperplasia,[26] EC with squamous differentiation,[27] EC treated with progestin,[30] and tamoxifen-associated EC[31]).

For the "index test" domain, three studies were considered at unclear risk of bias (criteria for β-catenin evaluation not completely clarified); high concerns were found for two studies (cytoplasmic expression of β-catenin lumped together with nuclear expression in the results).[21,22]

For the "reference standard" domain, neither significant risks of bias nor concerns about applicability were found.

For the "flow and timing" domain, two studies were considered at unclear risk of bias, because causes of the exclusion of some patients from immunohistochemical[23] or molecular analysis[20] were not specified.

All the remaining judgments were "low risk of bias."

Results of risk of bias assessment are summarized in Figure 2.

Nuclear expression of β-catenin showed a pooled sensitivity of 0.88 (95% CI, 0.81-0.93), with minimal heterogeneity among studies ($I^2 = 17.8\%$). Pooled specificity was 0.85 (95% CI, 0.81-0.88) with moderate heterogeneity ($I^2 = 59.9\%$). Pooled positive and negative likelihood ratios were 4.57 (95% CI, 3.04-6.87) and 0.20 (95% CI, 0.14-0.30), respectively, with moderate heterogeneity ($I^2 = 53.4\%$) and no heterogeneity ($I^2 = 0\%$), respectively Figure 3. Pooled DOR was 27.16 (95% CI, 12.87-57.33), with minimal heterogeneity ($I^2 = 3.5\%$). The overall diagnostic accuracy was high, with an AUC of 0.91 Figure 4.

Discussion

Our study showed that nuclear expression of β-catenin was an accurate immunohistochemical surrogate of *CTNNB1* exon 3 mutations in EC. To our knowledge, this may be the first systematic review and meta-analysis on this topic.

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Table 1

Characteristics of the Included Studies

Study	Country	Setting	Period of Enrollment	Inclusion Criteria	Sample Size	Histology	Sampling Method	Antibody Manufacturer	IHC Scoring Method	DNA/RNA Extraction	Molecular Analysis	Exclusions (Reasons)
Fukuchi et al[21]	Japan	National Cancer Center Hospital (Tokyo)	NR	All endometrial cancers	76	64 EAs (40 G1, 10 G2, 14 G3), 12 non-EAs (AdSq, CCC, SC, CS)	Hysterectomy	Transduction Laboratories	Any marked nuclear/ cytoplasmic	Fresh tissue, microdissection	PCR + sequencing	No exclusions
Kobayashi et al[22]	Japan	Sapporo Medical University	NR	All endometrial cancers	35	35 AdCs (*CTNNB1*-mt with 1 AdSq and 4 EAs [G1])	Hysterectomy	Transduction Laboratories	Any marked nuclear/ cytoplasmic	Fresh-frozen tissue	PCR + SSCP + sequencing	IHC on 17 (tissue availability)
Nei et al[20]	Japan	Sapporo Medical University Hospital	NR	All endometrial cancers	30	30 AdCs (11 G1, 10 G2, 9 G3)	Hysterectomy	Transduction Laboratories	Intensity grading 0-3	unclear	RT-PCR + sequencing	Molecular on 20 (unclear)
Ikeda et al[23]	Japan	Tohoku University Hospital	NR	All endometrial cancers	38	36 AdCs (22 G1, 9 G2, 5 G3), 1 AdSq G2, 1 CCC	Hysterectomy	Transduction Laboratories	Any striking nuclear	Fresh-frozen tissue	PCR + SSCP + sequencing	IHC on 9 (unclear)
Moreno-Bueno et al[24]	Spain	Department of Pathology, La Paz Hospital, Madrid	NR	Synchronous ovarian EA	5	5 EAs	Hysterectomy	Transduction Laboratories	Any nuclear	Microdissection	PCR + SSCP + sequencing	No exclusions
Saegusa et al[25]	Japan	Kitasato University Hospital	1988-1999	All endometrial cancers	199	199 EAs (128 G1, 39 G2, 32 G3)	Hysterectomy	Transduction Laboratories	Intensity grading 0-3, labeling index	Microdissection	RT-PCR + sequencing	Molecular on 71 (based on Southern blot results)
Ashihara et al[26]	Japan	Sapporo Medical University Hospital	NR	Coexisting endometrial hyperplasia	20	20 EAs (16 G1, 3 G2, 1 G3)	Hysterectomy	Transduction Laboratories	Intensity grading 0-2	Microdissection	PCR + sequencing	No exclusions
Ashihara et al[27]	Japan	Sapporo Medical University Hospital	NR	Squamous differentiation	35	35 EAs (7 G1, 23 G2, 5 G3)	Hysterectomy	Transduction Laboratories	Unclear	Microdissection	PCR + sequencing	No exclusions
Moreno-Bueno et al[28]	Spain	Hospital La Paz in Madrid; Hospital Santa Creu i San Pau, Barcelona	NR	All endometrial cancers	128	95 EAs, 15 SCs, 5 CCCs, 13 mixed	Hysterectomy	Transduction Laboratories	Percentage ≥5% (every case ≥5%)	Microdissection	PCR + sequencing	3 excluded
Machin et al[29]	Spain	Hospital Santa Creu i Sant Pau, Barcelona	NR	All endometrial cancers	73	59 EAs (21 G1, 21 G2, 17 G3), 5 SCs, 3 CCCs, 6 mixed	Hysterectomy	Transduction Laboratories	Any nuclear (every case >10%)	Fresh-frozen tissue, microdissection	PCR + SSCP + sequencing	No exclusions
Saegusa et al[30]	Japan	Kitasato University Hospital	1988-2000	Progestin therapy	23	23 EAs (G1)	Biopsy	Transduction Laboratories	Labeling index	Microdissection	PCR/RT-PCR + sequencing	5 excluded
Prasad et al[31]	United States	Memorial Sloan-Kettering Cancer Center	NR	Tamoxifen associated	58	46 EA, 6 SC, 6 CS	NR	Transduction Laboratories	Staining score 0-12	Microdissection	PCR + sequencing	No exclusions
Wappenschmidt et al[32]	Germany	University Hospitals of Kiel, Bonn, and Würzburg	NR	All endometrial cancers	82	62 EAs (16 G1, 35 G2, 11 G3), 6 SCs, 5 CSs, 3 SqCs, 3 MucCs, 2 CCCs, 1 undifferentiated carcinoma	hysterectomy	Transduction Laboratories	Intensity grading 0-3, labeling index	Microdissection	PCR + SSCP + sequencing	IHC on 22 (tissue availability)
Guerra et al33	Italy	S.Orsola-Malpighi Hospital, Bologna	NR	Synchronous ovarian cancer	11	5 EAs (2 G1, 3 G2), 2 SCs, 2 CCCs, 2 undifferentiated carcinoma	Hysterectomy	Novocastra	Only nuclear, nuclear/ membranous, only membranous	Microdissection	PCR + sequencing	No exclusion
Kim et al12	United States	University of Texas MD Anderson Cancer Center	Since 2000	All endometrial cancers	345	239 EAs (30 G1, 161 G2, 48 G3), 50 mixed, 47 non-EAs	Hysterectomy	BD Biosciences	Any nuclear (every case >5%)	Microdissection	NGS	IHC on 99 (53 *CTNNB1*-mt and 46 wt random)

AdC, adenocarcinoma; AdSq, adenosquamous carcinoma; CCC, clear cell carcinoma; CS, carcinosarcoma; EA, endometrioid adenocarcinoma; G1, grade 1; G2, grade 2; G3, grade 3; IHC, immunohistochemistry; MucC, mucinous carcinoma; NGS, next-generation sequencing; NR, not reported; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase polymerase chain reaction; SC, serous carcinoma; SSCP, single-strand conformation polymorphism.

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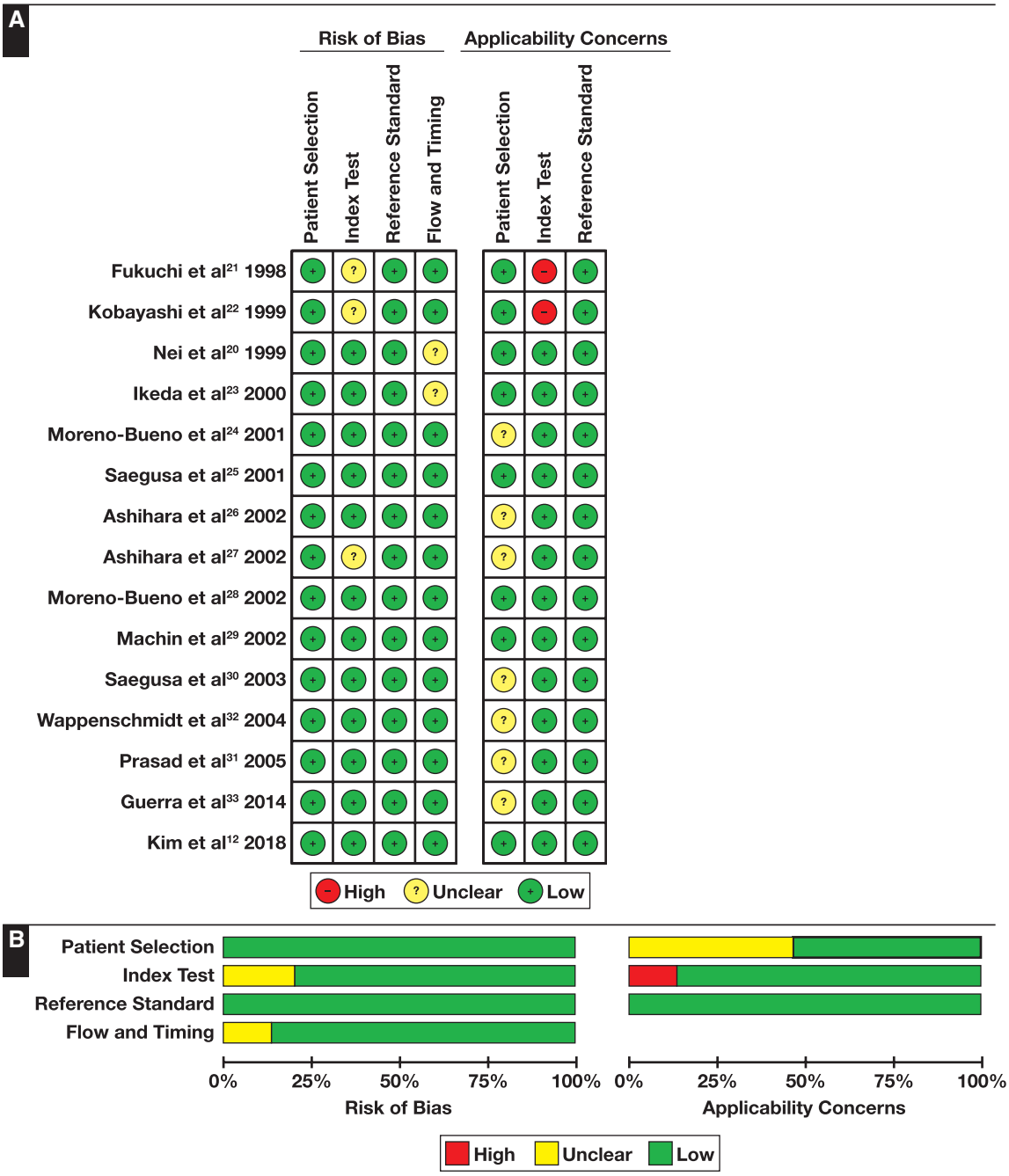

Figure 2 **A**, Assessment of risk of bias. Summary of risk of bias for each study. Plus sign: low risk of bias; minus sign: high risk of bias; question mark: unclear risk of bias. **B**, Risk of bias graph about each risk of bias item presented as percentages across all included studies.

Our results may implement the research for immunohistochemical surrogates of molecular analysis, indispensable to make applicable the TCGA classification of EC. In fact, this reclassification has been demonstrated to reduce under- and overtreatment of patients with EC but appears limited by prohibitive costs and technical complexity of analysis.[2] These problems should be solved to address the increased mortality of EC, which is now a global health problem.[1] Indeed, the major cause of this increased mortality seems to be linked to a risk stratification of EC based on histologic examination of surgical specimens. This examination is affected by poor reproducibility, even when assessed by expert pathologists.[4,5,34,35] Moreover, the poor reproducibility seems to regard the endometrium even more than other tissues.[36,37] β-Catenin is encoded by the *CTNNB1* gene

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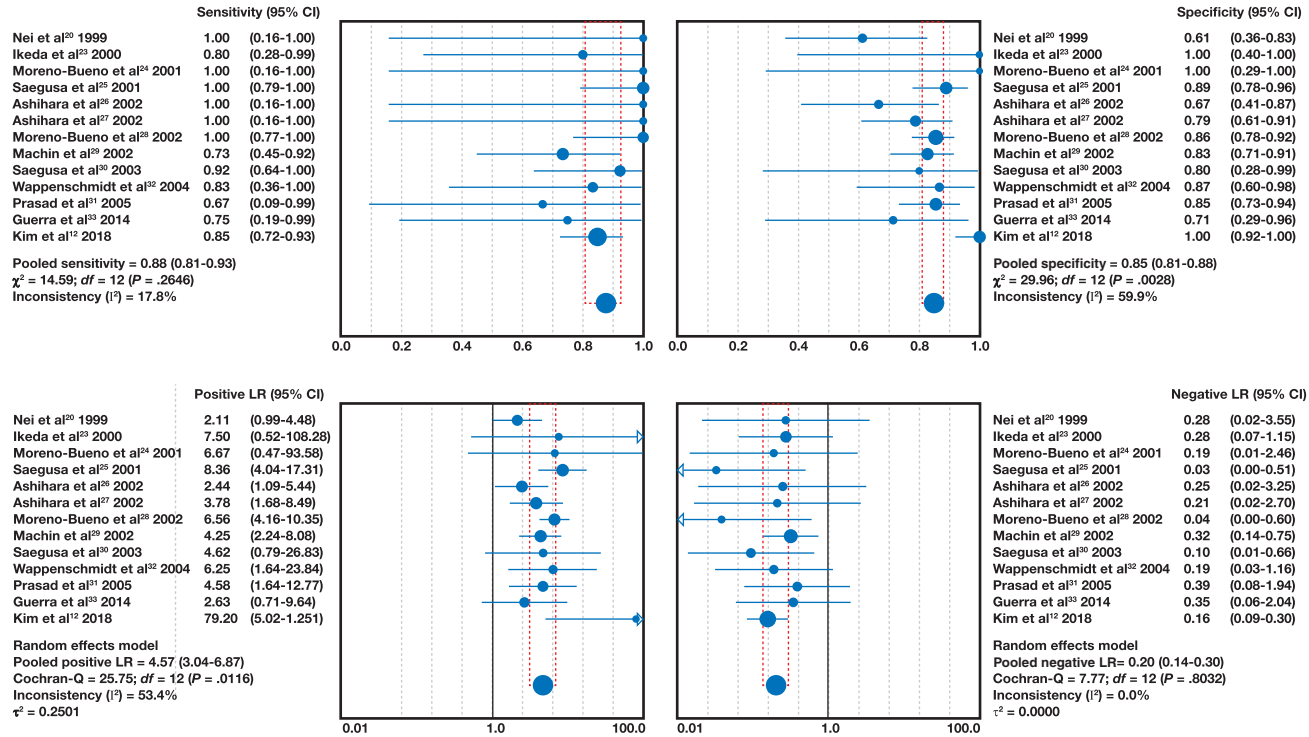

Figure 3 Forest plots reporting sensitivity, specificity, and positive and negative likelihood ratios for each study and as pooled estimates, with 95% confidence interval.

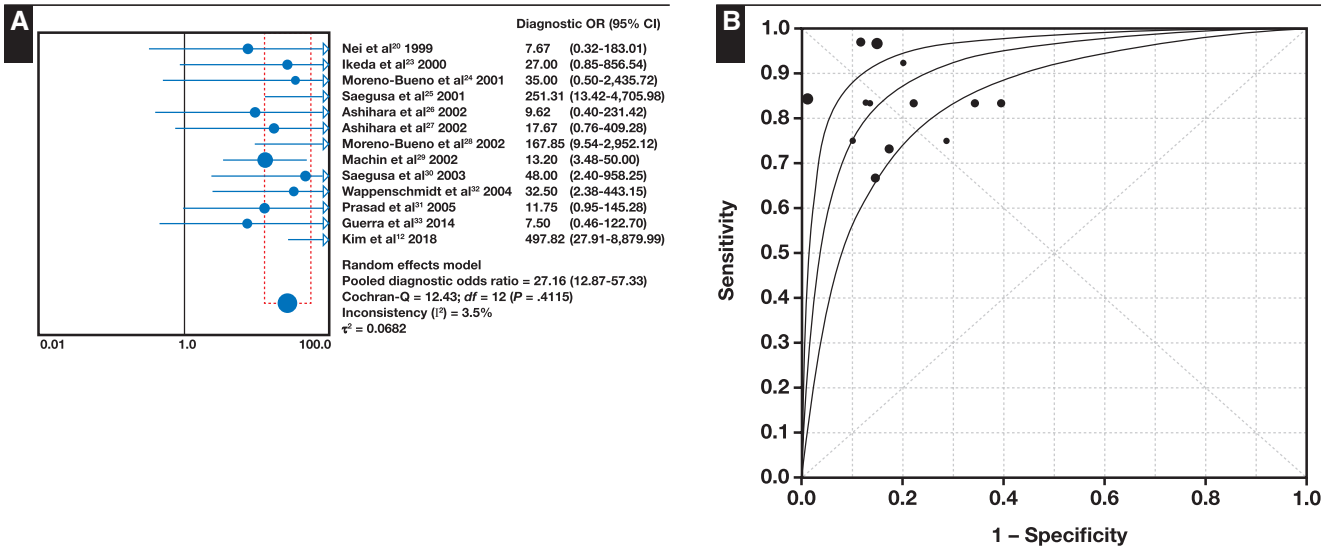

Figure 4 **A**, Forest plot reporting diagnostic odds ratio for each study and as pooled estimate, with 95% confidence interval. **B**, Area under the curve (AUC) calculated on summary receiver operating characteristic curves. AUC = 0.9088; SE = 0.0244. Cochran-Q = 0.8407; SE = 0.0268.

and is a key protein in Wnt signaling pathway. β-Catenin acts as a membrane link between cell-cell adherens junctions and cytoskeleton. Activation of the Wnt pathway makes β-catenin accumulate first into the cytoplasm and then into the nucleus, where it binds to transcription factors of the LEF/TCF family, favoring cell proliferation. Pathologic activation of this pathway can lead to cancerous transformation.[38,39]

As an oncogenic protein, β-catenin is known to be involved in endometrial carcinogenesis.[6,39] Mutations in

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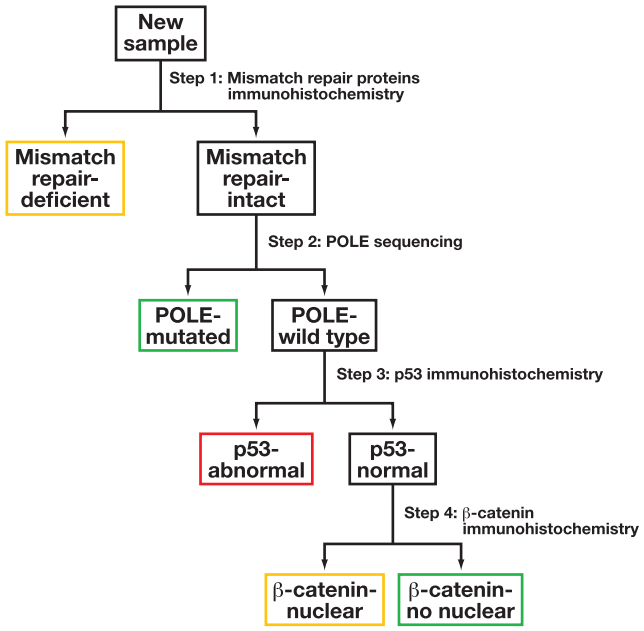

Figure 5 A possible integration of immunohistochemistry for β-catenin in the Proactive Molecular Risk Classifier for Endometrial Cancer as an additional fourth step. Green indicates good prognosis; yellow indicates intermediate prognosis; red indicates poor prognosis. POLE, polymerase-ε.

CTNNB1 occur in about half of ECs with a low mutational rate, a low somatic copy number alteration, and *TP53*-wt ("copy number–low group"), which constitute the largest group in the TCGA classification.[6] Contrary to the other groups, this group lacks a molecular signature and shows a heterogeneous prognosis.[6,8] An analysis of the Post Operative Radiation Therapy in Endometrial Carcinoma (PORTEC) cohort showed that mutations in exon 3 of *CTNNB1* identified a subset at higher risk within this group. In particular, in the absence of other prognostic factors, *CTNNB1* mutations made the prognosis switch from good to intermediate.[9] Other studies confirmed the independent prognostic significance of *CTNNB1* exon 3 mutations.[10,11] Therefore, some authors have considered *CTNNB1*-mt EC a separate molecular and prognostic group.[8]

Nuclear accumulation of β-catenin has been given interest as a possible immunohistochemical surrogate of *CTNNB1* mutations. However, its accuracy has never been defined. Recently, Kim et al[12] performed a molecular and immunohistochemical analysis of 99 ECs, showing that nuclear β-catenin had a sensitivity of 0.85 and a specificity of 1.00 in identifying *CTNNB1* exon 3 mutations. They were the first authors to perform such an analysis using NGS. They hypothesized the use of β-catenin immunohistochemistry as a screening text, followed by *CTNNB1* exon 3 sequencing only in negative or ambiguous cases.[12] Regarding sensitivity, the value they found (0.85) was quite close to our result (0.88), lying in our 95% CI (0.80-0.87). However, the specificity observed in our analysis appeared lower (0.85 vs 1.00). Such a difference may be due to the higher sensitivity of NGS in detecting mutations compared with older techniques.[40] In previous studies, some EC specimens might have been considered false positive (nuclear β-catenin expression and *CTNNB1*-wt) only because the pre-NGS sequencing technique did not detect the mutation.

The reliability of nuclear expression of β-catenin as a surrogate of *CTNNB1* mutations was not so obvious. In fact, nuclear accumulation of β-catenin may also occur in the absence of *CTNNB1* mutations, as can be observed in breast cancer.[41] Evidence supports that the association between *CTNNB1* mutations and nuclear expression of β-catenin is tissue specific and influenced by the expression of several other proteins.[12] Therefore, this association needed to be defined with specific regard to EC tissue.

Our study demonstrates that the association between nuclear β-catenin and *CTNNB1* mutations is particularly strong in EC. We found that nuclear β-catenin was an accurate immunohistochemical surrogate of *CTNNB1* mutations, with an AUC of over 0.90. Such result might support the clinical applicability of immunohistochemistry for β-catenin. As the risk stratification in EC is expected to radically change in the near future, the β-catenin immunostaining pattern might be considered in a prognostic algorithm, together with other significant factors, such as lymphovascular space invasion and immunohistochemical expression of p53, mismatch repair proteins, and L1CAM. Such an algorithm might be crucial in guiding adjuvant therapy, substantially reducing both under- and overtreatment.[9] To date, the best-known prognostic algorithm is the Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE), which involves immunohistochemistry for mismatch repair proteins, POLE sequencing, and p53 immunohistochemistry; these three steps allow defining four prognostic groups that superimpose on the four TCGA groups.[2,7] In this regard, immunohistochemistry for β-catenin might be introduced as an additional fourth step in the p53-normal group (which basically overlaps with the copy number–low TCGA group), identifying a subgroup at worse prognosis. A hypothetical integration of β-catenin immunohistochemistry in the ProMisE is shown in **Figure 5**.

Although our results appeared promising, there are some concerns to address. In particular, there are no standardized criteria for interpretation of β-catenin immunostaining. Regarding the percentage of stained cells, it seems that even a minimal percentage of

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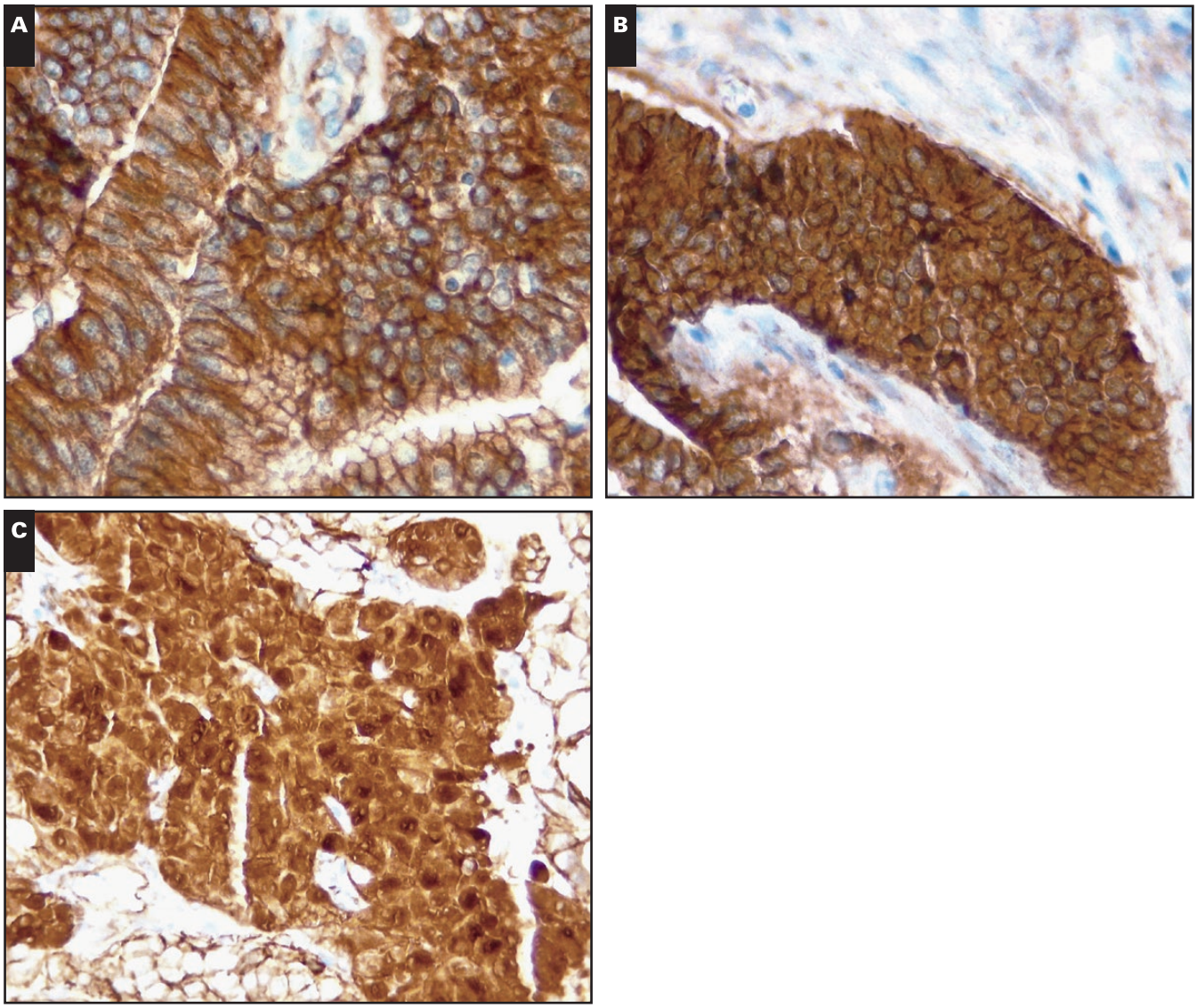

Image 1 Different patterns of β-catenin immunohistochemical expression (×600). **A**, Absence of nuclear expression (no nuclear brown staining). **B**, Weak nuclear expression (light brown nuclear nuance). **C**, Moderate to strong nuclear expression (brown to dark brown nuclear staining).

β-catenin–positive nuclei should be considered significant; indeed, *CTNNB1*-mt ECs may show even only few β-catenin–stained nuclei.[12] With respect to intensity of immunostaining, weak nuclear expression, appearing as a light brown nuance in the nucleus, may be observed in normal endometrium in the proliferative phase.[20] Therefore, it seems that only a nuclear expression at least moderate—appearing as a striking brown staining at immunohistochemistry—should be considered significant, as suggested by several authors.[21-23] On the other hand, weak nuclear expression might be considered nonsignificant or at least ambiguous **Image 1**.

Our results might also be affected by differences in methods of tissue withdrawal (eg, fresh tissue or microdissected tissue) for DNA extraction, as well as in the sensitivity of sequencing analysis, as discussed above.

Future studies should confirm whether immunohistochemistry for β-catenin may be routinely used for the risk stratification of EC. For this purpose, the definition of standardized criteria for interpreting β-catenin immunostaining and the use of NGS as reference standard are advised.

Conclusions

Immunohistochemical nuclear expression of β-catenin appears as an accurate surrogate of *CTNNB1* exon 3 mutations; therefore, it might be used as a less expensive

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prognostic marker in EC. Recent evidence suggests that the accuracy of β-catenin immunohistochemistry may be even higher if NGS is used as reference standard, with a virtual specificity of 100%.

Further studies are necessary to confirm the prognostic value and the clinical applicability of immunohistochemistry for β-catenin in the risk stratification in EC. For this purpose, standardized criteria for the interpretation of immunostaining should be defined, and NGS should be used as reference standard.

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