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AHNAK2 is a novel diagnostic biomarker for gallbladder adenocarcinoma

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Summary. Objective. To investigate the pathological diagnostic value of AHNAK2 in gallbladder carcinoma (GBC), especially in adenocarcinoma (AC).

Methods. Tissue microarrays (TMAs) were constructed from 296 gallbladder tumor cases, comprising 562 cores that included normal/atypical epithelium, low-grade intraepithelial neoplasia (LGIN), high-grade intraepithelial neoplasia/carcinoma *in situ* (HGIN/TIS), and GBC. Immunohistochemical staining for AHNAK2 and IMP3 was performed on these TMAs and another 10 GBC cases, and the sensitivity and specificity of AHNAK2 were assessed across different gallbladder tumor types.

Results. AHNAK2 immunohistochemical expression demonstrated a progressive increase across pathological stages ($p<0.001$). Among tumor types, AHNAK2 positivity was observed in 67.53% (260/385) of ACs, 97.83% (45/46) of adenosquamous/squamous cell carcinomas (ASC/SCCs), 34.78% (8/23) of neuroendocrine carcinomas/mixed neuroendocrine-non-neuroendocrine neoplasms (NEC/miNEN), but not in areas of NECs, and none in undifferentiated carcinomas (UCs). Importantly, among three grades of well, moderately, and poorly differentiated AC, the positive rate of AHNAK2 decreased from 70.59% (24/34), 70.11% (190/271), to 57.50% (46/80); conversely, IMP3 increased from 58.82%, 78.97% to 83.75%. Given the extremely low positivity rates of AHNAK2 and IMP3 in normal/atypical epithelium, combining these markers significantly improved diagnostic performance, demonstrating 83.73% sensitivity and 91.38% specificity for HGIN/TIS and GBC, achieving sensitivities of 91.18%, 90.77%, and 93.75% across well, moderately, and poorly differentiated ACs.

Conclusion. AHNAK2 demonstrates moderate sensitivity and high specificity in the pathological diagnosis of GBC, particularly for well-differentiated ACs. Combining AHNAK2 with IMP3 significantly enhances diagnostic sensitivity, achieving up to 90% across all AC grades.

Key words: Gallbladder carcinoma, Adenocarcinoma, Tumor grading, Diagnosis, AHNAK2, Biomarkers, Immunohistochemistry

Introduction

Gallbladder carcinoma (GBC) is the most common malignant tumor of the biliary system (Lau et al., 2017), with high malignancy and poor prognosis (Lai and Lau, 2008). GBC includes various pathological types and exhibits pathological heterogeneity. There are some diagnostic challenges in the pathological diagnosis (Sasaki and Sato, 2021). It is difficult to differentiate reactive atypia of the gallbladder epithelium from dysplasia, and similarly challenging to distinguish dysplasia involving Rokitansky-Aschoff sinuses from infiltrating adenocarcinomas (ACs). As well-differentiated AC has relatively regular glandular morphology, mild cellular atypia, and a low proliferative index, differentiating it from adenomyomatosis and reactive atypia of the epithelium associated with cholecystitis is often tricky. Additionally, some unique structures in the gallbladder, such as the Luschka duct, are located deep in the serosal layer and usually must be distinguished from neoplastic lesions. As for biopsy specimens, sometimes

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Abbreviations. AC, adenocarcinoma; ASC, adenosquamous carcinoma; GBC, gallbladder carcinoma; HGIN, high-grade intraepithelial neoplasia; ICPN, intracholecystic papillary neoplasm; IHC, immunohistochemistry; LGIN, low-grade intraepithelial neoplasia; miNEN, mixed neuroendocrine-non-neuroendocrine neoplasm; NEC, neuroendocrine carcinoma; OS, overall survival; ROC, Receiver Operating Characteristic; SCC, squamous cell carcinoma; TIS, carcinoma *in situ*; TMA, tissue microarray; UC, undifferentiated carcinoma.

the tissue may be small with significant crush or burn artifacts, making diagnosis extremely challenging.

Several immunohistochemical markers, including CD10, p53, and IMP3, have been utilized to distinguish benign from malignant biliary tract lesions, yet each presents limitations (Tretiakova et al., 2012; Ghosh et al., 2013; Carrasco et al., 2021). Although loss of CD10 expression may aid in diagnosing bile duct biopsies (Tretiakova et al., 2012), its presence in 44.2% of gallbladder cancers limits its specificity (Tretiakova et al., 2012). While p53 shows increasing abnormal expression from reactive lesions (10.3%) to dysplasia (50%) (Sica et al., 2024), its sensitivity in malignancies remains suboptimal (49-49.4%) (Mikata et al., 2021; Sano et al., 2022). IMP3 exhibits improved sensitivity (69.2-87.7%) and specificity (86%) (Shi et al., 2013a; Sasaki and Sato, 2021), yet it fails to detect approximately 30% of well-to-moderately differentiated ACs, precisely the most diagnostically challenging cases (Kim et al., 2020). This significant gap underscores the need for more reliable diagnostic markers.

AHNAK2, identified in 2004 as a high-molecular-weight protein (>600 kD) (Komuro et al., 2004), shows elevated expression in multiple cancers, including lung, thyroid, pancreatic, renal, and bladder malignancies, but is minimally expressed in normal tissues (Zardab et al., 2022). However, existing evidence derives largely from sequencing or omics studies, and immunohistochemical validation remains limited to small cohorts of breast, colorectal, and esophageal ACs (Xu et al., 2022).

Based on the high expression of AHNAK2 in pancreatic cancer (Zardab et al., 2025) and the morphological and immunophenotypic similarities between pancreatic cancer and GBC, we hypothesize that AHNAK2 may also be highly expressed in GBC and serve as a diagnostic marker. This study, therefore, evaluates AHNAK2 expression in a large gallbladder tumor cohort and compares its performance with IMP3, focusing on its utility in distinguishing benign from malignant lesions, particularly in AC.

Materials and methods

Case selection

The inclusion criteria for this study were as follows: Patients who underwent surgery and were pathologically diagnosed with gallbladder tumors at Fudan University Zhongshan Hospital were selected between November 2013 and September 2018. These cases must have sufficient tissue samples available. Additionally, patients should not have any other malignancies that could affect prognosis, nor should they have received neoadjuvant therapy. In total, 296 cases for tissue microarrays (TMAs) and 10 cases containing both carcinoma and high-grade intraepithelial neoplasia/carcinoma *in situ* (HGIN/TIS) in the same slide were included in this study. All participants provided signed informed consent.

Clinical data collection

We collected the clinical and pathological data of patients, including gender, age at initial onset, time of surgery, surgical procedure, pathological type, tumor differentiation, and clinical stage. Follow-up was conducted via electronic medical record and telephone. Overall survival (OS) was measured from the date of surgery to the date of death due to disease. The last follow-up date was August 28th, 2020. Deaths from other causes were recorded as lost to follow-up.

This study was approved by the Ethics Committee of Fudan University Zhongshan Hospital (B2019-200R).

Pathological diagnoses

The cases and tissue core diagnoses were classified according to the WHO Classification of Tumors of the Digestive System (5th edition). HGIN/TIS includes intracholecystic papillary neoplasm (ICPN) with HGIN/TIS, adenoma with HGIN/TIS, and flat HGIN/TIS; low-grade intraepithelial neoplasia (LGIN) includes ICPN with LGIN, adenoma with LGIN, and flat LGIN. Grading of gallbladder AC was based on the degree of glandular differentiation (Burt et al., 2018).

Construction of tissue microarrays

The high-output TMA technique was used to construct TMAs (Shi et al., 2013b). For a single case, tissue cores were taken separately if there were multiple types of tumors distinguishable, such as AC and squamous cell carcinoma (SCC) areas in adenosquamous carcinoma (ASC) or the ICPN area and AC area in ICPN-related invasive AC. For these reasons, the number of tissue cores in the TMA exceeded the number of cases.

A total of 562 tissue cores from 296 patients were included in the study. Malignant tumors accounted for 82.92% (466/562), including AC (385, 68.51%), ASC/SCC (46, 8.19%), NEC/miNEN (23, 4.09%), and undifferentiated carcinoma (UC) (12, 2.14%). HGIN/TIS accounted for 6.76% (38/562), including ICPN with HGIN (22, 3.91%), adenoma with HGIN (7, 1.25%), and flat HGIN (9, 1.60%). LGIN accounted for 3.56% (20/562), including ICPN with LGIN (5, 0.89%), adenoma with LGIN (8, 1.42%), and flat LGIN (7, 1.25%). Normal/atypical epithelium accounted for 6.76% (38/562).

Among the 385 AC tissue cores, well-differentiated, moderately differentiated, and poorly differentiated subtypes accounted for 8.83% (34/385), 70.39% (271/385), and 20.78% (80/385), respectively.

Immunohistochemistry (IHC) procedures

AHNAK2 (clone F3G4C10, 1:200, Jusbio Science (Shanghai) Co., Ltd) and IMP3 (clone EPR12021, 1:100, Abcam) immunohistochemical staining were performed

on the Roche Ventana automated staining system. Positive reactions for AHNAK2 and IMP3 are indicated by brown staining of the tumor cell membrane and/or cytoplasm, with a clean background and no nonspecific staining (AHNAK2 exhibits strong positive staining in nerve fibers, which may be utilized as a reliable internal positive control). The expression level is assessed based on the intensity and extent of positivity, recorded as the percentage of positive cells plus the intensity of positivity (e.g., 80%++). The optimal cut-off value was calculated based on the Receiver Operating Characteristic (ROC) curve, with any staining above 1% considered positive and less than 1% considered negative.

Statistical methods

The χ^2 test was used to analyze categorical data. The student's t-test was employed to compare means between two groups for parametric data. The McNemar test was used to compare detection rates among different groups. The Wilcoxon matched-pairs signed rank test was used to compare maker expression in HGIN/TIS and carcinoma in the same case. Kaplan-Meier analysis was applied to calculate patient survival times, and Cox proportional hazards regression analysis was used to assess the impact of various factors on prognosis. The sensitivity and specificity of AHNAK2 and IMP3 for diagnosing GBC and HGIN/TIS were compared using ROC curves. A *p*-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 22.0.

Results

Association between AHNAK2 and clinicopathological characteristics in 296 gallbladder tumor patients

A total of 296 patients with gallbladder tumors were included in the TMA cohort, and the clinical pathological characteristics and AHNAK2 expression are summarized in Table 1. A total of 18 (6.08%) were preinvasive tumors (11 HGIN/TIS and 7 LGIN), while 278 (93.92%) were invasive carcinoma. Two hundred and one (67.91%) tested positive for AHNAK2, while 95 (32.09%) tested negative. No statistically significant association was found between AHNAK2 expression and gender or age. AHNAK2 expression was significantly associated with pathological classification. Positive expression rates were 14.29% (1/7) for LGIN, 45.45% (5/11) for HGIN/TIS, 69.13% (159/230) for AC, 100% (27/27) for ASC/SCC, 56.25% (9/16) for NEC/miNEN, and 0% for UC ($p<0.001$). A significant correlation was identified between AHNAK2 expression and clinical staging ($p<0.001$). Positive expression rates by stage were as follows: 28.57% (8/28) for stage 0-I, 61.76% (42/68) for stage II, 75.00% (90/120) for stage III, and 76.25% (61/80) for stage IV. AHNAK2 expression also showed statistical significance with the surgical approach. The AHNAK2-positive rate was 76.67% (69/90) in the non-radical surgery group and 64.08% (132/206) in the radical surgery group ($p=0.033$).

Survival information was obtained for 274 patients, with a median survival time of 33 months. The OS rates at 1, 2, 3, and 5 years were 70.5%, 55.3%, 48.1%, and 43.2%, respectively. Univariate analysis revealed a significantly worse prognosis in AHNAK2-positive patients compared with AHNAK2-negative counterparts ($p=0.002$). The AHNAK2-positive group demonstrated a median survival time of 24 months with a 5-year OS rate of 37.3%, whereas the AHNAK2-negative group had a median survival time non-reached and a 5-year OS rate of 55.3%. However, AHNAK2 failed to demonstrate prognostic stratification value across different clinical stages.

AHNAK2 expression in 562 TMA cores

Among the 562 tissue cores, 336 were positive for AHNAK2, while 226 were negative. AHNAK2 typically shows cytoplasmic and membranous positivity in GBC cells (Fig. 1). The positivity rates for AHNAK2 in normal/atypical epithelium, LGIN, HGIN/TIS, and GBC were 5.26% (2/38), 15.00% (3/20), 47.37% (18/38), and

Table 1. AHNAK2 expression in gallbladder tumors (296 cases).

Clinical-pathological characteristics	Total	AHNAK2		*p*-value
		Positive	Negative	
Age (years)				0.494
≤63	141	93 (65.96%)	48 (34.04%)	
>63	155	108 (69.68%)	47 (30.32%)	
Gender				
female	176	117 (66.48%)	59 (33.52%)	0.524
male	120	84 (70.00%)	36 (30.00%)	
Pathological classification				**<0.001**
LGIN	7	1 (14.29%)	6 (85.71%)	
HGIN/TIS	11	5 (45.45%)	6 (54.55%)	
AC	230	159 (69.13%)	71 (30.87%)	
ASC/SCC	27	27 (100%)	0	
NEC/miNEN	16	9 (56.25%)	7 (43.75%)	
UC	5	0	5 (100%)	
Clinical Stage				
0	18	5 (27.78%)	13 (72.22%)	**<0.001**
Stage I	10	3 (30.00%)	7 (70.00%)	
Stage II	68	42 (61.76%)	26 (38.24%)	
Stage III	120	90 (75.00%)	30 (25.00%)	
Stage IV	80	61 (76.25%)	19 (23.75%)	
Type of Surgery				
Radical	206	132 (64.08%)	74 (35.92%)	**0.033**
Non-radical	90	69 (76.67%)	21 (23.33%)	
Total	296	201	95	

AC, adenocarcinoma; ASC/SCC, adenosquamous/squamous cell carcinomas; HGIN/TIS, high-grade intraepithelial neoplasia/carcinoma *in situ*; LGIN, low-grade intraepithelial neoplasia; NEC/miNEN, neuroendocrine carcinomas/mixed neuroendocrine-non-neuroendocrine neoplasms; UC, undifferentiated carcinoma.

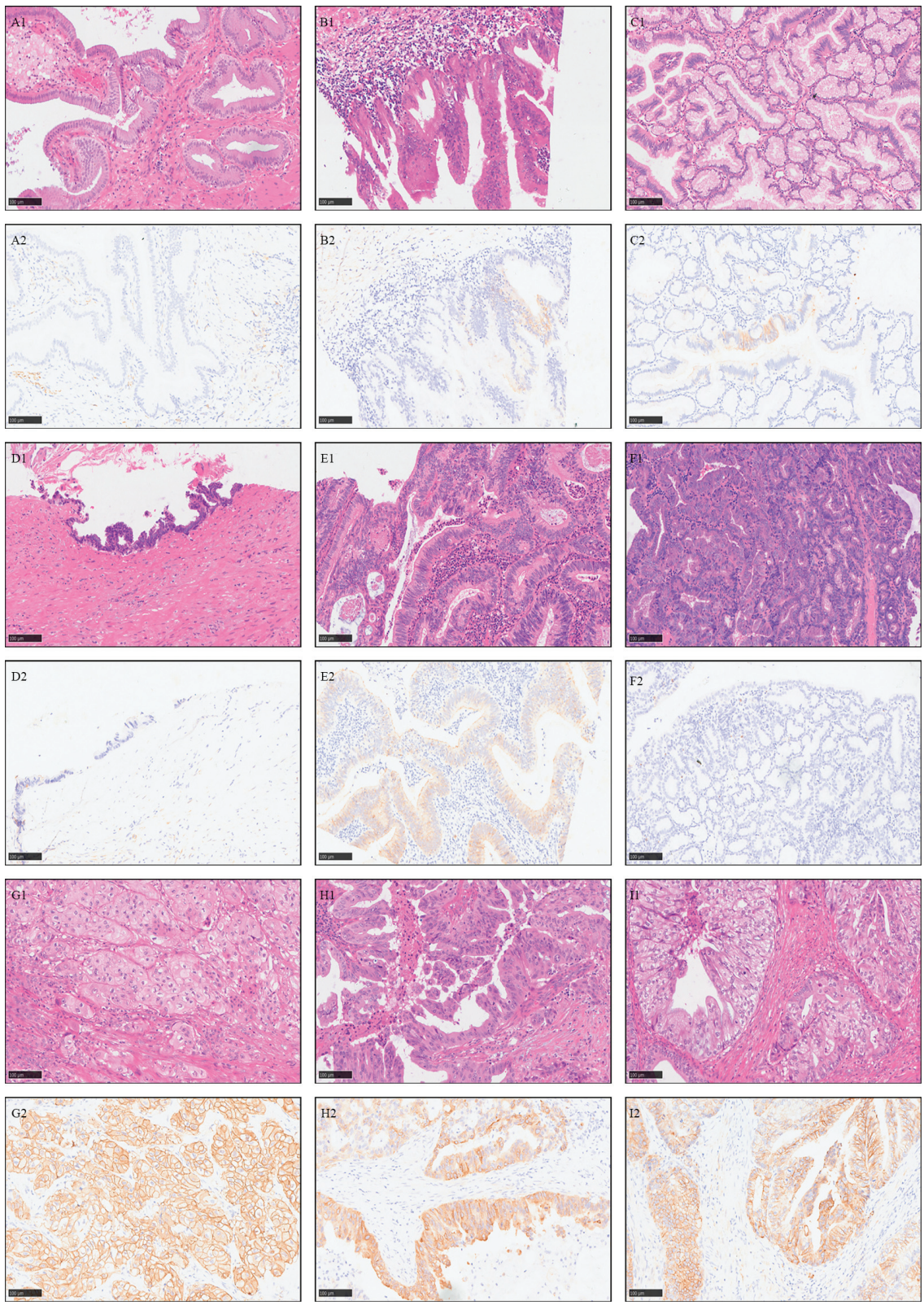

Fig. 1. AHNAK2 expression in different gallbladder lesions. **A1, A2.** AHNAK2 is negative in normal gallbladder epithelium. **B1, B2.** AHNAK2 is typically negative in atypical epithelium, with focal weak staining observed in rare cases. **C1, C2.** AHNAK2 is typically negative in LGIN, with focal weak staining observed in rare cases. **D1-F2.** In flat HGIN/TIS **(D1, D2)**, ICPN with HGIN/TIS **(E1, E2)**, adenoma with HGIN/TIS **(F1, F2)**, the positivity of AHNAK2 is 47.37%. **G1-I2.** In most GBC cases **(G1, H1, I1)**, AHNAK2 shows positive expression with specific brown membranous and/or cytoplasmic staining of tumor cells **(G2, H2, I2)**, against a clean background free of nonspecific staining. Scale bars: 100 μm.

67.17% (313/466), respectively (p<0.001) (Table 2, Fig. 1). Diagnostic performance analysis revealed AHNAK2 had an area under the curve (AUC) of 0.785 (95% confidence interval (CI): 0.734-0.837) for distinguishing malignant/premalignant lesions (GBC/HGIN/TIS) from non-malignant lesions (normal/atypical epithelium/LGIN). The sensitivity for diagnosing GBC and HGIN/TIS was 65.67%, and the specificity was 91.38%. These data indicate that AHNAK2 has moderate sensitivity and high specificity for diagnosing GBC and HGIN/TIS.

We further investigated the positivity rate of AHNAK2 across various histological types of GBC (Table 2). The positivity rate for AHNAK2 was 67.53% (260/385) in ACs and 97.83% (45/46) in ASC/SCCs. In NEC/miNEN, the positivity rate was 34.78% (8/23), with most pure NEC areas being negative, while non-neuroendocrine tumor areas were positive. It was fully negative in UC (0/12).

Since the pathological diagnosis of well-differentiated AC is the most challenging, we analyzed the positive rate of AHNAK2 in ACs with different grades and compared it with IMP3. The AHNAK2 positivity rate was highest in well-differentiated AC (70.59%, 24/34), followed by moderately differentiated (70.11%, 190/271) and poorly differentiated AC (57.50%, 46/80) (Table 3, Fig. 2).

IMP3 expression in 562 TMA cores

Among the 562 tissue cores, IMP3 immunoreactivity was detected in 403 cases, with 159 showing negative expression. IMP3 positivity demonstrated a significant stepwise increase along the neoplastic progression spectrum (Table 2): 5.26% (2/38) in normal/atypical epithelium, 15.00% (3/20) in LGIN, 60.53% (23/38) in HGIN/TIS, and 80.47% (375/466) in GBC (p<0.001). Diagnostic performance analysis revealed that IMP3 had

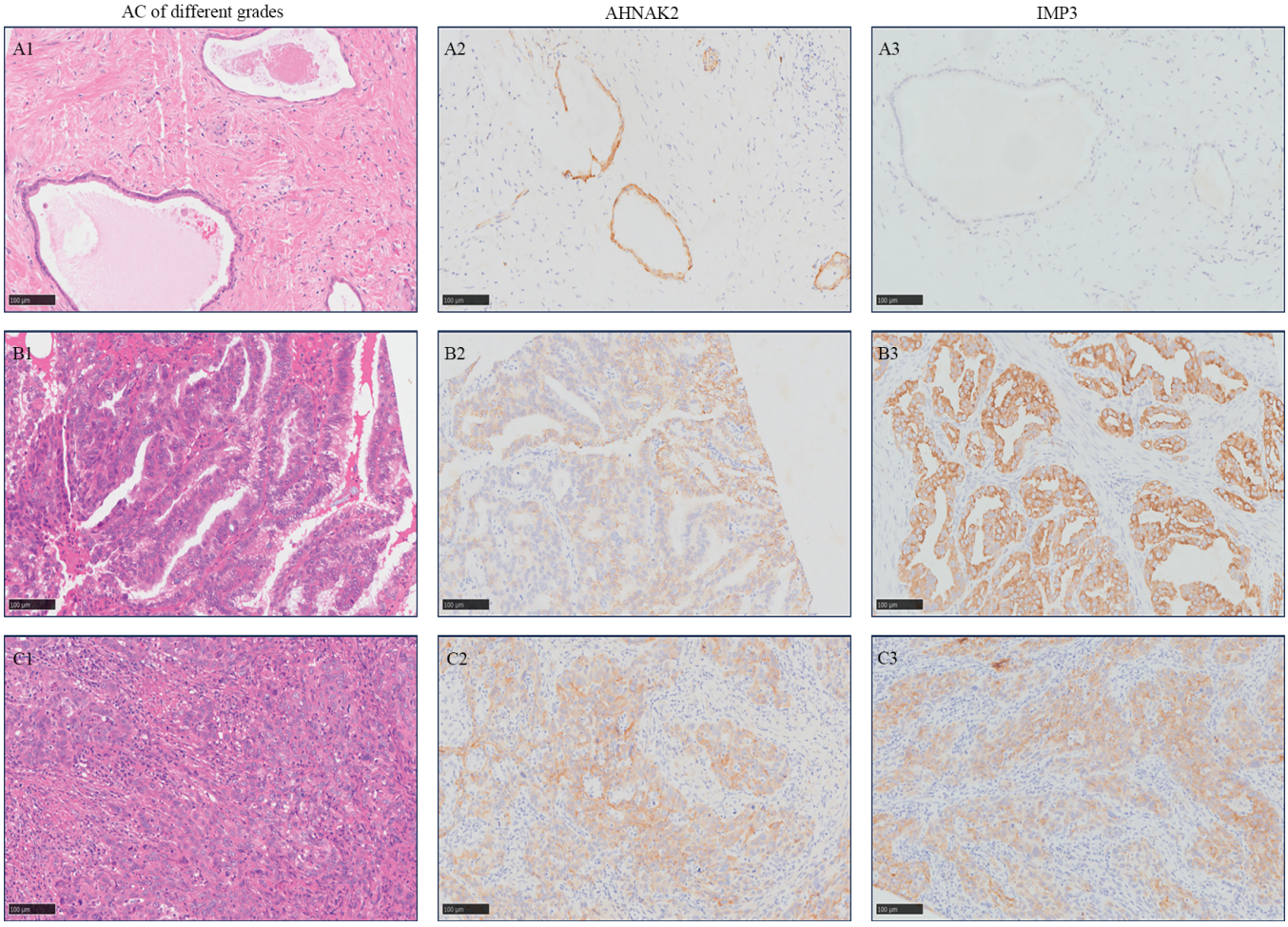

Fig. 2. Differential expression patterns of AHNAK2 and IMP3 in gallbladder AC by histological grade. **A1-A3.** Well-differentiated AC **(A1)** demonstrates strong AHNAK2 positivity **(A2)** with absent IMP3 expression **(A3)**. **B1-B3.** Moderately-differentiated AC **(B2)** shows co-expression of AHNAK2 **(B2)** and IMP3 **(B3)**. **C1-C3.** Poorly-differentiated AC **(C1)** exhibits retained AHNAK2 **(C2)** and IMP3 **(C3)** immunoreactivity. Scale bars: 100 μm.

an AUC of 0.869 (95% CI: 0.837-0.907) for distinguishing malignant/premalignant lesions (GBC/HGIN/TIS) from non-malignant lesions (normal/atypical epithelium/LGIN). The test demonstrated 91.38% specificity and 78.97% sensitivity.

We further investigated the positivity rate of IMP3 across various histological types of GBC (Table 2). The positivity rate was 78.18% (301/385) in ACs, 91.30% (42/46) in ASC/SCCs, 96.96% (20/23) in NEC/miNEC, and 100% in UC.

We evaluated IMP3 expression patterns across different grades of AC (Table 3, Fig. 2). IMP3 immunopositivity demonstrated a progressive increase with tumor dedifferentiation: well-differentiated ACs showed the lowest positivity rate (58.82%, 20/34), moderately-differentiated ACs exhibited intermediate expression (78.97%, 214/271), while poorly-differentiated tumors displayed the highest IMP3 positivity (83.75%, 67/80).

The combined use of AHNAK2 and IMP3 significantly enhances the sensitivity of GBC diagnosis, particularly in well-differentiated AC

Given the different positivity rates of AHNAK2 and IMP3 across various types and grades of GBC, where AHNAK2 exhibits a decreasing positivity rate and IMP3 shows an increasing positivity rate with poorer differentiation, we evaluated the combined use of both markers in different gallbladder tumor types, with a positive result defined as positivity in either marker. The combined positivity rate was 10.53% (4/38) in normal/atypical epithelium, 30.00% (6/20) in LGIN, 73.68% (28/38) in HGIN/TIS, and 92.49% (431/466) in GBC.

The combined use of AHNAK2 and IMP3 achieved a sensitivity of 83.73% and a specificity of 91.38% for detecting HGIN/TIS and GBC (Fig. 3). The combination significantly enhanced the sensitivity of gallbladder AC diagnosis: Overall sensitivity was 91.43%, representing a 13.25% increase compared with IMP3 alone. For different grades of AC, the sensitivity improvements

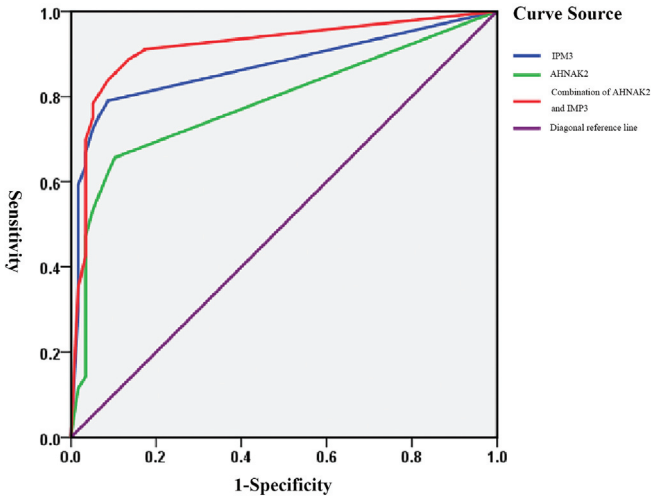

Fig. 3. The diagnostic value comparison of AHNAK2, IMP3, and their combination for HGIN/TIS and GBC. The AUC of AHNAK2 was 0.785 (95% CI: 0.734-0.837), with a diagnostic sensitivity of 65.67% and specificity of 91.38%. IMP3 showed an AUC of 0.869 (95% CI: 0.837-0.907), with a sensitivity of 78.97% and specificity of 91.38%. The combination of AHNAK2 and IMP3 achieved an AUC of 0.916 (95% CI: 0.879-0.954), demonstrating a sensitivity of 83.73% and specificity of 91.38%.

Table 2. AHNAK2 and IMP3 expression in gallbladder tumors (562 TMA cores).

Classification	Total	AHNAK2		IMP3		*p*-value
		Positive	Negative	Positive	Negative	
Normal/Atypical	**38**	**2 (5.26%)**	**36 (94.74%)**	**2 (5.26%)**	**36 (94.74%)**	**1**
LGIN	**20**	**3 (15.00%)**	**17 (85.00%)**	**3 (15.00%)**	**17 (85.00%)**	**1**
ICPN with LGIN	5	0	5 (100.00%)	0	5 (100.00%)	
Adenoma with LGIN	8	1 (12.50%)	7 (87.50%)	0	8 (100%)	
Flat LGIN	7	2 (28.57%)	5 (71.43%)	3 (42.86%)	4 (57.14%)	
HGIN/TIS	**38**	**18 (47.37%)**	**20 (52.63%)**	**23 (60.53%)**	**15 (39.47%)**	**0.25**
ICPN with HGIN	22	13 (59.09%)	9 (40.91%)	14 (63.64%)	8 (36.36%)	
Adenoma with HGIN	7	2 (28.57%)	5 (71.43%)	2 (28.57%)	5 (71.43%)	
Flat HGIN	9	3 (33.33%)	6 (66.67%)	7 (77.78%)	2 (22.22%)	
GBC	**466**	**313 (67.17%)**	**153 (32.83%)**	**375 (80.47%)**	**91 (19.53%)**	**<0.001**
AC	385	260 (67.53%)	125 (32.47%)	301 (78.18%)	84 (21.82%)	
ASC/SCC	46	45 (97.83%)	1 (2.17%)	42 (91.30%)	4 (8.70%)	
NEC/miNEC	23	8 (34.78%)	15 (65.22%)	20 (86.96%)	3 (13.04%)	
UC	12	0	12 (100.00%)	12 (100%)	0	
Total	**562**	**336**	**226**	**403**	**159**	

AC, adenocarcinoma; ASC/SCC, adenosquamous/squamous cell carcinomas; HGIN/TIS, high-grade intraepithelial neoplasia/carcinoma *in situ*; ICPN, intracholecystic papillary neoplasm; LGIN, low-grade intraepithelial neoplasia; NEC/miNEN, neuroendocrine carcinomas/mixed neuroendocrine-non-neuroendocrine neoplasms; UC, undifferentiated carcinoma.

were as follows: well-differentiated increased to 91.18% (a 32.35% increase), moderately-differentiated increased to 90.77% (an 11.81% increase), and poorly-differentiated increased to 93.75% (a 10.0% increase) compared with IMP3 alone (Fig. 4).

Comparative analysis of AHNAK2 and IMP3 expression in carcinoma and HGIN/TIS lying in the immediate vicinity

AHNAK2 and IMP3 expression were evaluated in 10 cases containing both carcinoma and HGIN/TIS in the same slide (Fig. 5). In carcinoma regions, AHNAK2 positivity was observed in 90% of cases (9/10), with the staining extent ranging from 60 to 100%. IMP3 was positive in 100% of carcinoma cases (10/10), with staining extent varying between 20 and 100%. In well-differentiated ACs, IMP3 exhibited limited staining extent and weak intensity, a pattern that appeared complementary to AHNAK2 expression. In HGIN/TIS regions, AHNAK2 positivity was detected in 50% of cases (5/10), with staining extent ranging from 5 to 80%, predominantly showing weak intensity. IMP3 was positive in 70% of HGIN/TIS cases (7/10), with staining extent ranging from 50 to 100% and frequently demonstrating moderate to strong intensity. The positivity rate of AHNAK2 was significantly higher in carcinoma than in HGIN/TIS (p=0.004). Although IMP3 also showed a higher positivity rate in carcinomas compared with HGIN/TIS, but this difference did not reach statistical significance (p=0.118).

Discussion

This study demonstrates, for the first time, the diagnostic utility of AHNAK2 in distinguishing benign and malignant gallbladder tumors. Using IHC on a spectrum of tissues from normal/reactive atypical epithelium to LGIN, HGIN/TIS, and GBC, we established that AHNAK2 exhibits moderate sensitivity and high specificity in differentiating HGIN/TIS/GBC from LGIN or benign reactive conditions. Notably, its positive rate in well-differentiated gallbladder AC surpassed that of the established biomarker, IMP3. The combination of AHNAK2 with IMP3 significantly enhanced diagnostic sensitivity for GBC and HGIN/TIS while maintaining high specificity.

Identified in 2004, AHNAK2 is a molecular analog

Table 3. AHNAK2 and IMP3 expression in ACs of different grades.

AC differentiation	Total	AHNAK2		IMP3		*p*-Value
		positive (%)	negative (%)	positive (%)	negative (%)	
well-differentiated	34	24 (70.59%)	10 (29.41%)	20 (58.82%)	14 (41.18%)	0.31
moderately-differentiated	271	190 (70.11%)	81 (29.89%)	214 (78.97%)	57 (21.03%)	0.018
poorly-differentiated	80	46 (57.50%)	34 (42.50%)	67 (83.75%)	13 (16.25%)	<0.001
Total	385	260 (67.53%)	125 (32.47%)	301 (78.18%)	84 (21.82%)	0.001

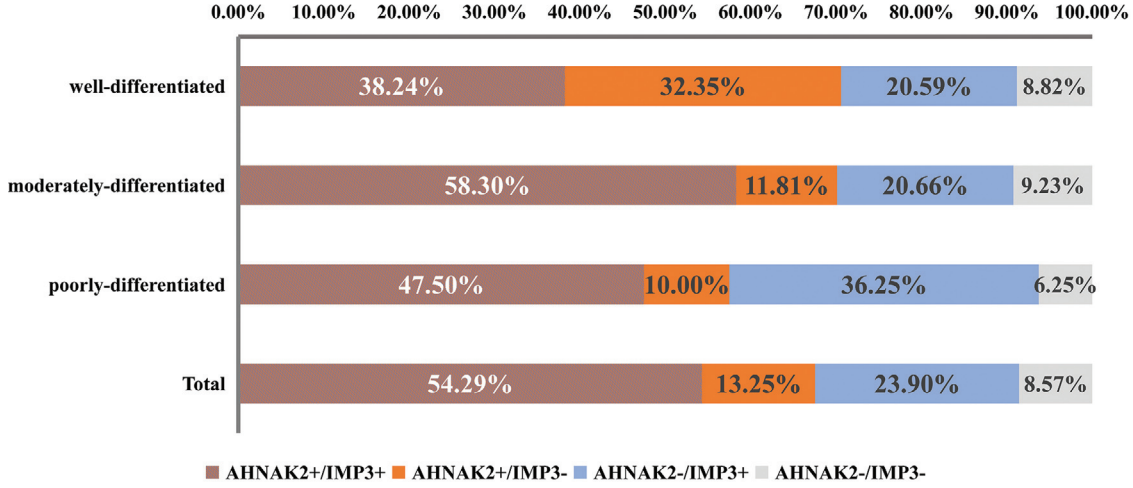

Fig. 4. Sensitivity comparison of AHNAK2, IMP3, and their combined use in AC by differentiation grade. The combined marker panel (AHNAK2+IMP3) significantly improved sensitivity *versus* IMP3 alone: Well-differentiated AC: 91.18% vs. 58.83% (Δ+32.35%); Moderately-differentiated AC: 90.77% vs. 78.96% (Δ+11.81%); Poorly-differentiated AC: 93.75% vs. 83.75% (Δ+10.00%); All ACs combined: 91.44% vs. 78.19% (Δ+13.25%).

of AHNAK (Komuro et al., 2004). The latter, a giant nuclear protein of approximately 629 kDa, was first characterized in 1992 and derives its name from the Hebrew word for "giant" (Shtivelman et al., 1992). Current understanding of AHNAK2 remains limited. It is broadly expressed in tissues, including the brain, squamous epithelium, smooth muscle, and neurons, with potential roles in disease pathogenesis, though definitive mechanistic evidence remains scarce (Vinci et al., 2022). AHNAK2 demonstrates upregulated expression across multiple malignancies, including thyroid, lung, pancreatic, renal, and bladder cancers (Zardab et al., 2022), where its overexpression correlates with poor prognosis. Existing evidence predominantly derives from sequencing or omics analyses, with only limited IHC validation supporting its diagnostic utility in breast cancer, colorectal cancer, esophageal AC (Xu et al., 2022), thyroid papillary carcinoma (Zhang et al., 2025), and pancreatic ductal AC (Lu et al., 2017). However, no prior studies have reported its expression profile in GBC. In this study, a progressive upregulation pattern emerged across pathological progression: 15.00% positivity in LGIN, 47.37% in HGIN/TIS, and 67.17% in GBC. This constitutes the first documentation of AHNAK2's ascending expression gradient spanning the gallbladder carcinogenesis continuum (normal/atypical epithelium → LGIN → HGIN/TIS → GBC). Furthermore, this study represents the first comprehensive analysis of AHNAK2 expression across various tumor subtypes, suggesting its potential role in tumor differentiation and maintenance of both squamous and glandular differentiation pathways.

AHNAK2 and IMP3 demonstrate complementary diagnostic value, with their combined use significantly improving the sensitivity of GBC diagnosis. Compared to IMP3, a well-established biomarker for biliary tract tumors, AHNAK2 shows comparable specificity but slightly lower sensitivity. Our study reveals complementary expression patterns: AHNAK2 shows minimal positivity in UCs and NEC, where IMP3 exhibits higher positivity rates, while AHNAK2 displays strong expression in well-differentiated gallbladder ACs but weak expression in poorly differentiated ACs, a pattern inverse to IMP3. Thus, the combination of AHNAK2 and IMP3 enhances diagnostic sensitivity while maintaining high specificity. This dual-marker approach also shows markedly improved sensitivity across tumor differentiation grades: sensitivities for well,

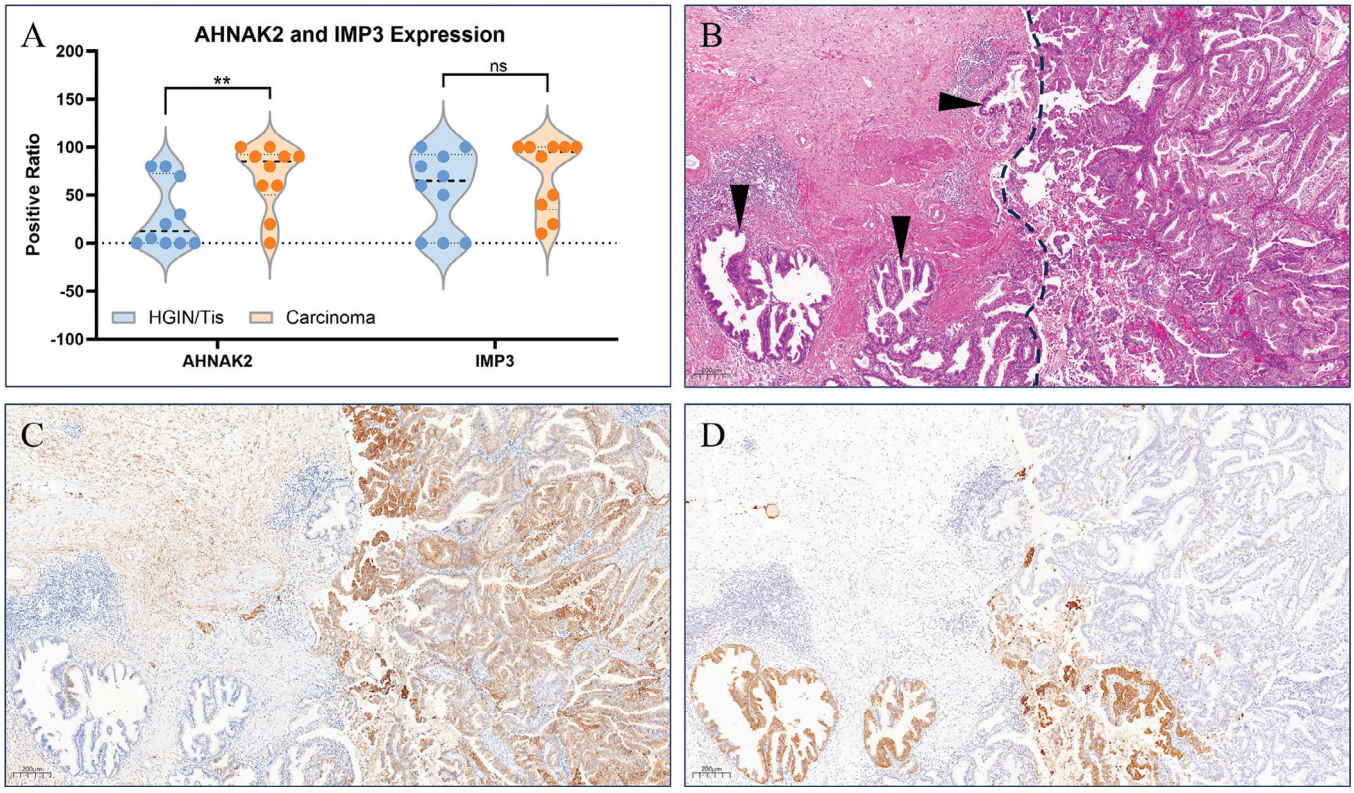

Fig. 5. AHNAK2 and IMP3 expression on the tissue sections with invasive carcinomas and HGIN/TIS lying in the immediate vicinity. **A.** AHNAK2 and IMP3 positive ratio in carcinoma and HGIN/TIS. **B.** Representative H&E of HGIN/TIS and AC in the same case. The left side of the image shows HGIN/TIS (indicated by the arrow), while the right side shows AC. **C.** AHNAK2 was positive in AC, while only focal and weakly positive in HGIN/TIS. **D.** IMP3 showed focal positivity in AC, while positive in HGIN/TIS. Scale bars: 200 μm.

moderately, and poorly differentiated ACs reach 91.18%, 90.77%, and 93.75%, respectively, representing improvements of 32.35%, 11.81%, and 10.0%, over IMP3 alone. The AHNAK2/IMP3 combination demonstrates superior sensitivity, specificity, and practicality compared with previous biomarker panels (Shi et al., 2013a). Earlier studies employed a four-marker panel (maspin, IMP3, S100P, and pVHL) for GBC diagnosis, where pVHL negativity combined with positivity for ≥two other markers showed 90.3% sensitivity for ACs *versus* no staining in benign/reactive cases (Shi et al., 2013a). In contrast, our two-marker panel achieves comparable diagnostic performance (83.73% sensitivity, 91.38% specificity for HGIN/TIS and GBC, 91.43% sensitivity for AC) with more straightforward interpretation and better cost-effectiveness.

High AHNAK2 expression shows a positive correlation with clinical staging of GBC, demonstrating increased positivity rates in advanced-stage cases and indicating poor prognosis in patients with elevated AHNAK2 levels. These findings suggest that AHNAK2 could be involved in GBC progression. Similar correlations have been reported in lung AC, where high AHNAK2 expression positively associates with clinical stage, T-stage, and N-stage, showing elevated levels in patients with larger tumor volumes and lymph node metastasis (Zheng et al., 2021). In differentiated thyroid carcinoma, increased AHNAK2 expression also correlates with advanced clinical staging (Xu et al., 2024). The clinical relevance of AHNAK2 as a prognostic marker requires further validation.

Despite identifying AHNAK2 as a promising diagnostic biomarker for GBC in a large cohort, this study has several limitations. Notably, the number of cases in the normal/reactive epithelium, LGIN, and HGIN/TIS groups was relatively limited, which may affect the reliability of the diagnostic performance. The research was conducted on TMAs and only 10 tissue sections with invasive malignancies and HGIN/TIS lying in the immediate vicinity, thus the findings may not adequately reflect tumor heterogeneity. Moving forward, we plan to validate the diagnostic utility of AHNAK2 in clinical gallbladder tumor specimens and biopsy samples. Furthermore, its real-world clinical applicability will need to be confirmed through larger multicenter trials. In parallel, comprehensive *in vivo* and *in vitro* studies will be essential to elucidate the functional roles and molecular mechanisms of AHNAK2 in gallbladder carcinogenesis.

In summary, we identified AHNAK2 as a promising diagnostic biomarker for GBC with subtype-specific expression. AHNAK2 and IMP3 showed complementary diagnostic value in pathological evaluation. Their combined application significantly enhanced diagnostic sensitivity for GBC while maintaining specificity. The diagnostic value of AHNAK2, both alone and in combination with IMP3, requires further validation in real-world clinical samples.

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Conflicts of Interest. The authors disclose that they have no significant relationships with, or financial interest in, any commercial companies pertaining to this article.

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