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Original Article

Morphological and immunophenotypical analysis of the spindle cell component in adenomyomatous hyperplasia of the gallbladder

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Summary

Background. Adenomyomatous hyperplasia (AMH) of the gallbladder, reported in 1-8.7% of cholecystectomies, consists of cystically dilated sinuses/glands with a surrounding spindle cell proliferation which is thought to be composed of smooth muscle cells. Myofibroblasts are contractile cells that secrete a variety of biochemical modulators causing a "field-effect". Myofibroblasts can be immunohistochemically distinguished from smooth muscle cells by their desmin negativity.

Methods. Eighteen cases of AMH and five cases each of chronic follicular cholecystitis, chronic cholecystitis, gallbladder carcinoma and 10 colonic diverticular disease were stained with actin and desmin. The percentage of myofibroblasts was estimated by the difference between actin and desmin staining in the same field. Statistical anlysis was performed using SPSS 22.0.

Results. The percentage of actin staining was significantly higher in AMH and gallbladder carcinoma compared to chronic follicular and chronic cholecystitis ($p = 0.04$). The percentage of desmin staining did not show any significant difference between the four groups. The estimated myofibroblastic population was significantly higher in AMH when compared to chronic follicular and chronic cholecystitis ($p = 0.005$).

Conclusion. The spindle cell proliferation around cystically dilated glands in AMH is composed predominantly of myofibroblasts and of smooth muscle cells as previously described. This finding suggest a derangement in epithelial-stromal interactions as the underlying pathophysiology in AMH.

Key words: adenomyomatous hyperplasia, myofibroblast, gallbladder, adenomyoma, digital pathology

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Conflict of interest
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Introduction

Adenomyomatous hyperplasia (AMH) of the gallbladder, reported in 1-8.7% of cholecystectomies [1-2], is characterized by epithelial proliferation into cystically dilated sinuses/glands with a surrounding spindle cell component which is thought to be made of hypertrophic smooth muscle cells [3,4].

The symptoms of AMH are non-specific and usually presents with right upper quadrant abdominal pain. AMH is commonly associated with gallstones and chronic cholecystitis [5,6].

The first case of AMH was reported by Jutras et al. in 1960 [3]. Jutra et al. described hyperplastic cholesteroloses of the gallbladder composed of

an epithelial proliferation and muscle hypertrophy of the gallbladder wall with an out-pouching of the mucosa into or through the thickened muscular layer. They later elaborated on this phenomenon, describing the distinct radiological, anatomical and histological features, renaming the entity adenomyoma and adenomyomatosis in 1966 [4].

Aldridge et al. first suggested AMH to be precancerous in nature [7]. Subsequent studies implicated AMH as a precursor of gallbladder cancer and reported a higher incidence of gallbladder carcinoma in segmental type AMH, one of the three described subtypes of AMH [8,9]. However, more recent studies have suggested that AMH is a non-neoplastic condition resulting from hyperplasia of the gallbladder wall and as such is unrelated to gallbladder carcinoma [10,11]. Reactive changes due to the chronic inflammatory milieu have been proposed as the etiologic factor behind AMH [1]. However, reports of cases of AMH occurring in the pediatric population, raise concerns for a developmental component; since AMH in infants and young children cannot be explained entirely by chronic inflammatory reaction [12,13].

The spindle cell component is a major component of AMH [14]. Proliferating smooth muscle can potentially obstruct glandular structures leading to their occlusion and progressive dilation. Furthermore, stromal cells, such as (myo)fibroblasts, are metabolically active with the secretion of bioactive molecules creating a bidirectional dialog with the proliferating epithelium [15]. However, to the date, the identity of the spindle cells in AMH has not been further characterized and quantified. In this study, we aim to further characterize the spindle cell component of this elusive entity. For practical purposes, the subepithelial spindle cells can be categorized in two possible cell lineages: a myofibroblastic one, which is positive for smooth muscle actin and negative for desmin and smooth muscle one, which is positive for both smooth muscle actin and desmin [16]. Using this principle, we aim to quantify the myofibroblastic proliferation in AMH and compare it with other neoplastic and non-neoplastic lesions of the gallbladder, in hope of gaining a deeper understanding of AMH.

Materials and methods

Case selection

This was a case control study approved by the Institutional Review Board (FWA00000176). A case search was performed using "CoPath", the pathology computerized database application at the Mount Sinai Medical Center, Department of Pathology. The search included all gallbladders diagnosed as "adenomyomatous hyperplasia" or "adenomyoma" in the period from January 1st, 2008- December 31st, 2018. Eighteen cases of AMH were reteived and included in the study. Five cases each of chronic cholecystitis, chronic follicular cholecystitis and gallbladder carcinoma were included as controls. In addition, 10 cases of colonic diverticular disease with and without inflammation and fibrosis were also stained for comparison.

Immunohistochemistry

The formalin-fixed paraffin-embedded blocks of the selected representative slides of cases and controls were stained for anti-actin smooth muscle (1A4) (Cell Marque, Rocklin, CA, USA; 1A4 clone) and anti-desmin (Ventana Medical Systems, Inc, Tucson, AZ, USA; DE-R-11clone) using the Ventana automated platform comprising of Ventana Benchmark automated slide stainer and UltraView Universal Alkaline Phosphatase Red Detection Kit (Ventana Medical Systems, Inc, Tucson, AZ, USA). ALK protein (Cell Marque, Rocklin, CA, USA) was evaluated in selected cases showing prominent spindle cell proliferation to rule out inflammatory myofibroblastic tumor. Appropriate positive and negative controls were run simultaneously.

Qualitative marker expression quantification

Subepithelial stromal spindle cell components surrounding areas of deeply seated glands representing lesional cells of AMH were reviewed by pathologists and selected for analysis. In the controls, the stroma surrounding Rokitansky-Aschoff sinuses was selected for the analysis. The percentage of spindle cell component staining for smooth muscle actin and desmin in the previously selected fields was estimated by two independent pathologists at a magnification of 400 x. The difference in the percentage of spindle cells staining for actin and desmin was calculated for each pathologist and the final percentage difference as calculated from each pathologists reading were averaged.

Digital pathology analysis

From each case and control, actin and desmin stained whole slides were scanned with a tile-based method on brightfield illumination and an objective of 5x using the Aperio LV1 scanner (Leica Biosystems, Inc, Buffalo Grove, IL, USA). Subsequent analysis was conducted on QuPath (V 0.2.0-m8, University of Edinburgh, UK). The area best representing the lesion were annotated as the Region Of Interest (ROI) (Fig. 1). Marker expression was quantified within the ROIs using the positive pixel count tool with the following parameters:

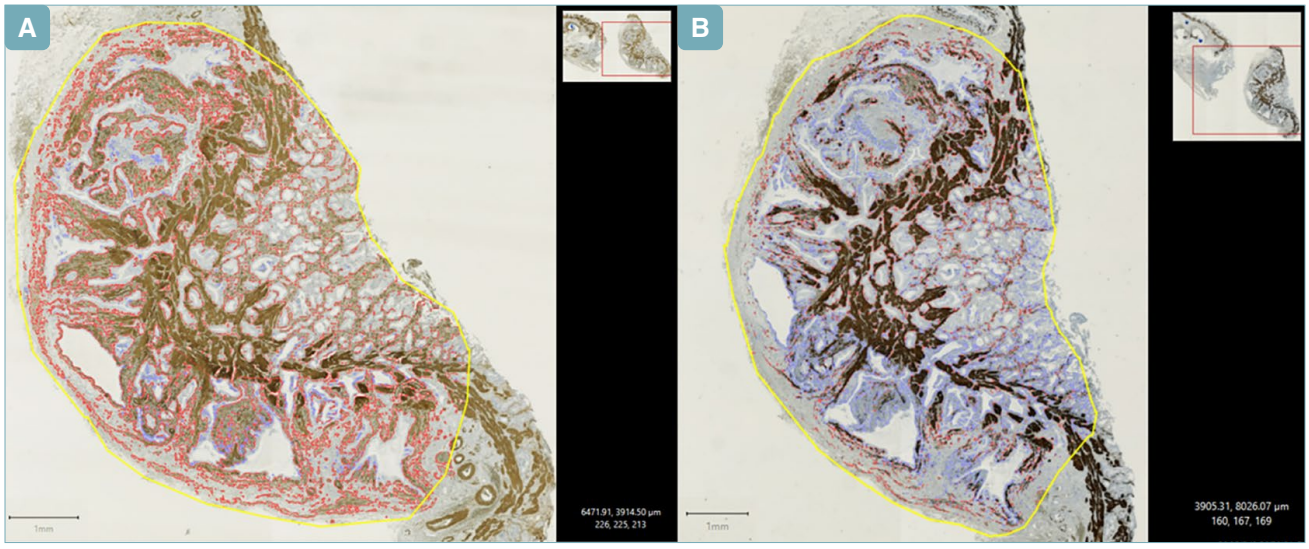

Figure 1. Example of quantification, by digital pathology analysis, of smooth muscle actin (A) and desmin (B) expression in a case of AMH. Whole scanned slides were obtained using an objective of 5x using the Aperio LV1 scanner (Leica Biosystems, Inc, Buffalo Grove, IL, USA) and processed in QuPath (V 0.2.0-m8, University of Edinburgh, UK). Regions of interest (ROIs) were selected in fields showing full thickness of the gallbladder wall with diagnostic findings. ROIs (yellow) were analyzed for positive (red) and negative (blue) pixel quantification. Bar: 1 mm.

downsample factor ranging from 4.0 to 11.0, Gaussian sigma of 2 µm, and a hematoxylin threshold (negative) of 0.19 OD units. The threshold of positivity was defined as the mean OD of positive stain in ROI minus one standard deviation. For most cases the positive threshold ranged from 0.19 up to 0.3 OD (Fig. 1).

Statistical analysis

Comparison of the percentage staining for actin and desmin in cases and controls was done using chi quare tests, independent median tests and Kruskal Wallis tests run on SPSS version 22.0. The percentage of spindle cells that were positive for actin but negative for desmin was taken as an estimate of myofibroblastic differentiation, while the percentage of spindle cells positive for both actin and desmin was taken as an estimate of smooth muscle differentiation. The interobserver agreement in assessing percentage of myofibroblastic population was assessed using Cohen's Kappa.

All procedures performed in the current study were approved by IRB and/or national research ethics committee (FWA00000176) in accordance with the 1964 Helsinki declaration and its later amendments. Formal written informed consent was not required with a waiver by the appropriate IRB and/or national research ethics committee.

Results

Eighteen cases of AMH and five cases each of chronic cholecystitis, chronic follicular cholecystitis and gallbladder carcinoma were retrieved from the archives of Mount Sinai Medical Center, Pathology department along with 10 cases of colonic diverticular disease with and without inflammation and fibrosis. The mean age of the study population was 64.88 ± 16.40 years. There was no significant difference in the age and gender distribution among the five groups. Fourteen of the 18 cases of AMH had concomitant cholelithiasis.

The percentage of spindle cells showing positive staining with actin was significantly higher in AMH and gallbladder carcinoma as compared to chronic follicular and chronic cholecystitis for both pathologists ($p = 0.04$). The percentage of spindle cells showing positive desmin staining was significantly lower in the AMH and gallbladder carcinoma groups ($p = 0.049$) as compared to chronic follicular and follicular cholecystitis for both the pathologists. (Fig. 2) The estimated myofibroblastic population was significantly higher in AMH and gallbladder carcinoma as compared to chronic follicular and chronic cholecystitis for both the pathologists ($p = 0.023$ and $p = 0.005$) taken separately as well as when averaged ($p = 0.000$).

The interrater agreement was low with a Cohen's Kap-

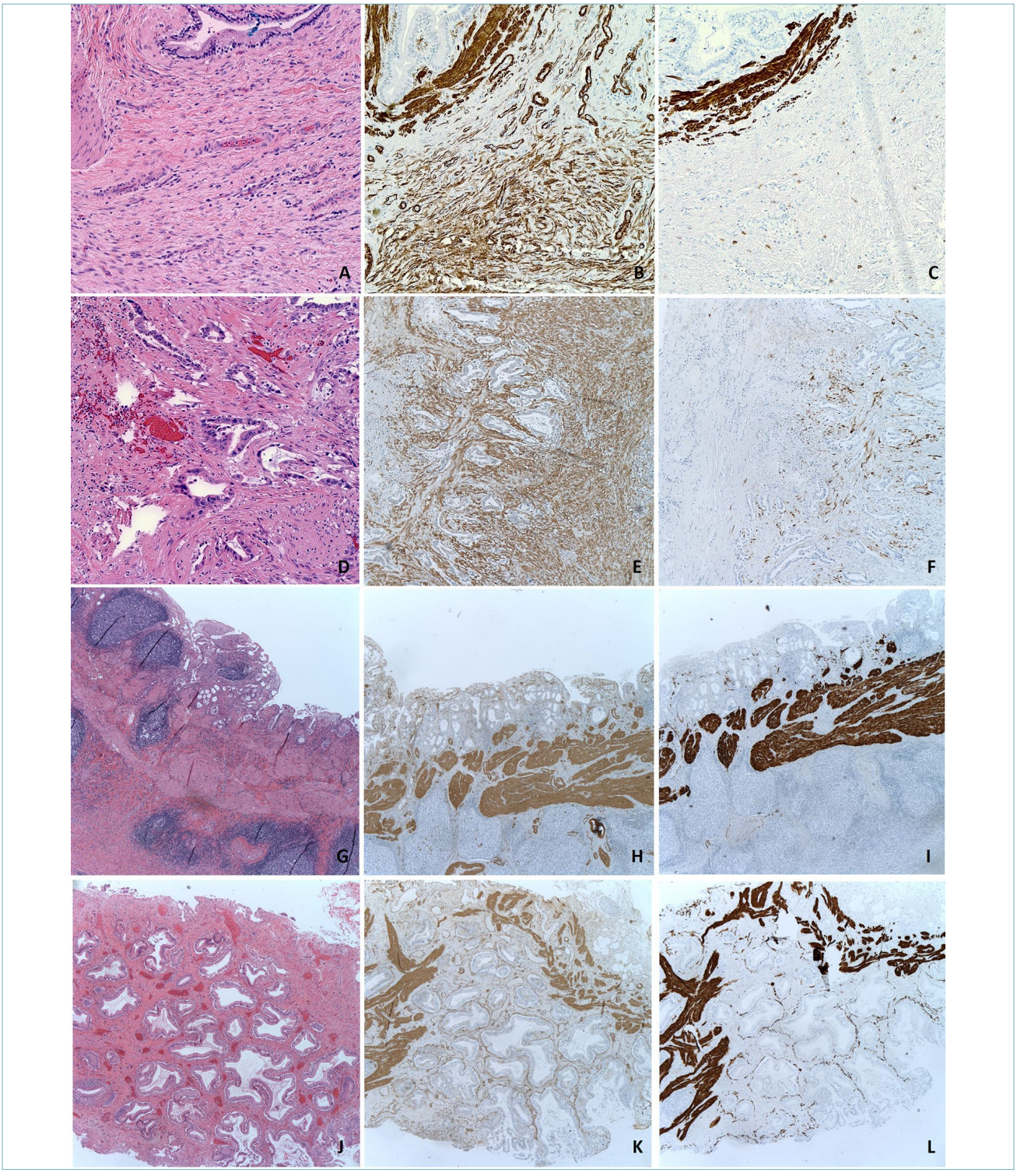

Figure 2. (A) Representative case of gallbladder with adenomyomatous hyperplasia (B) Actin staining in adenomyomatous hyperplasia (C) Desmin staining in adenomyomatous hyperplasia (D) Representative case of gallbladder with carcinoma (E) Actin staining in gallbladder carcinoma (F) Desmin staining in gallbladder carcinoma (G) Representative case of gallbladder with chronic follicular cholecystitis (H) Actin staining in chronic follicular cholecystitis (I) Desmin staining in chronic follicular cholecystitis (J) Representative case of gallbladder with chronic cholecystitis (K) Actin staining in chronic cholecystitis (L) Desmin staining in chronic cholecystitis.

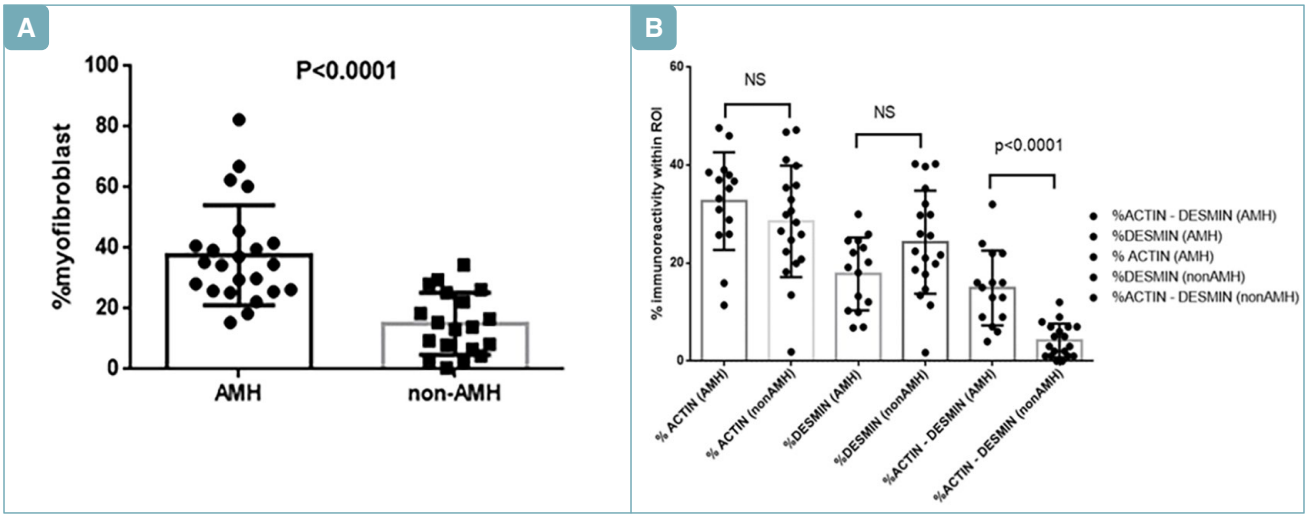

Figure 3. (A). Box plots showing percentage spindle cells with positivity for actin and desmin on digital quantification within ROI using the positive pixel count tool in AMH and non-AMH cases. (B). Box plots showing estimate myofibroblast percentage on digital quantification within ROI using the positive pixel count tool in AMH and non-AMH cases.

pa value of 0.291 compared across all cases. However, the interrater agreement (Cohen's Kappa) improved to 0.921 in cases where the difference in percentage of cells staining for actin and desmin was greater than 20% difference in actin and desmin staining were taken into account. All the cases of AMH were stained for ALK protein and were negative.

Additional ten cases of colonic diverticular disease in different stages of progression including diverticulosis, acute diverticulitis and perforated diverticulitis, were assessed for actin and desmin staining in the stromal spindle cells around the diverticuli and were uniformly found to have higher desmin positivity as compared to actin positivity.

On digital quantification within ROI using the positive pixel count tool, the percentage of spindle cells showing positive staining with actin was significantly higher in AMH as compared to chronic cholecystitis (p = 0.023) (Fig. 3A). The estimated myofibroblastic population was significantly higher in AMH as compared to chronic follicular, chronic cholecystitis and gallbladder carcinoma (p = 0.001). (Fig. 3B). These findings are summarized in Table I.

Table I. Comparison of subepithelial and transmural expression of smooth muscle actin and desmin in cases of AH and other gallbladder diseases.

Condition		% Subepithelial smooth muscle cells (actin+, desmin+)	% Subepithelial (myo)fibroblasts (actin+, desmin-)	% Transmural actin positivity in ROI	% Transmural desmin positivity in ROI	% Of transmural myofibroblasts (actin-desmin) in ROI
AH	Mean ± SD	21.4 ± 12.1	48.6 ± 17.7	33.7 ± 9.6	20.7 ± 9.2	39.4 ± 18.3
	Median (Range)	20 (3-45)	47.5 (20-88)	35.3 (11.4-47.6)	21.2 (6.8-40.3)	37.2 (2.1-82.4)
Chronic Follicular Cholecystitis	Mean ± SD	46.5 ± 26.4	13.0 ± 29.1	26.2 ± 9.0	23.2 ± 11.2	14.1 ± 15.2
	Median (Range)	55 (0-65)	0 (0-65)	22.3 (18.2-39.9)	18.6 (13.4-39.7)	6.6 (0.4-34.4)
Chronic cholecystitis	Mean ± SD	63.5 ± 18.5	4.0 ± 7.6	33.4 ± 9.7	29.6 ± 7.5	10.6 ± 4.8
	Median (Range)	65 (43-90)	0 (0-18)	35.4 (20.8-46.7)	29.9 (19.9-40.2)	9.4 (4.5-16.7)
Gallbladder carcinoma	Mean ± SD	5 ± 3.5	70.0 ± 10.9	18.7 ± 10.9	15.3 ± 8.7	16.2 ± 6.5
	Median (Range)	2.5 (3-10)	72.5 (53-80)	25.7 (1.9-26.6)	19.1 (1.7-22.4)	15.5 (8.0-25.7)
Statistical significance		p = 0.005	p = 0.000	P = 0.023	P = 0.11	P = 0.001

Discussion

Adenomyomatous hyperplasia (AMH) of the gallbladder is a relatively uncommon entity that presents with right upper abdominal pain and is reported in 1-8.7% of cholecystectomies [1-2]. Histological examination reveals AMH to be comprised of a glandular epithelial proliferation with cystically dilated glands surrounded by a spindle cell proliferation which is thought to be hypertrophic smooth muscle cells [3,4]. AMH was first described as a hyperplastic cholesterolosis by Jutras et al. in 1960 [3]. The authors reported an epithelial proliferation and hypertrophy of the muscles of the gallbladder wall with an out-pouching of the mucosa into or through the thickened muscular layer. Multiple reports and case series have been published since, but they fail to address the exact etiopathogenesis of this condition [12, 17-18].

Three morphological types of AMH have been described thus far. The fundal type, the most common type, is localized in the fundus of the gallbladder. The segmental type is annular and is found in the body of the gallbladder. The diffuse type, as the name suggests, is a generalised thickening of the gallbladder wall [19]. Though most of the cases of AMH in our study were of the fundal type, no distinction was made between the three types for the purpose of this study.

Katoh et al. in 1986 reported a rare case of noninvasive carcinoma of the gallbladder arising in the surface mucosa of localized type AMH and suggested a possible premalignant nature [20]. This theory was further propounded by Aldridge et al. in their report of a case of gallbladder adenocarcinoma occurring in localized adenomyomatosis [3]. Subsequent studies reported a higher incidence of gallbladder carcinoma in segmental type AMH and questioned the malignant potential of this entity [8,9]. Kai et al. concluded that although the accompanying gallstones and/or inflammation were more likely responsible for the carcinogenesis [21]. This theory is supported by multiple reports of high incidence of cholelithiasis in AMH [5,6].

The premalignant potential of AMH has been further disproved by studies such as by Xiao et al. in which they found no difference in the expression of Ki-67, P53, EGFR, survivin, and PCNA (genes implicated in gallbladder carcinoma) between chronic cholecystitis and AMH [11,21,22]. None of the cases of AMH in our study had any concomitant intraepithelial atypia or gallbladder carcinoma.

AMH is usually associated with cholelithiasis and chronic cholecystitis [5,6]. This association is seen in our study with 14 out of 18 cases having concomitant cholelithiasis. As such reactive changes due to a chronic inflammatory milieu have been proposed as the etiologic factor behind AMH [1]. Chronic stimulation by gallstones and cholecystitis leads to epithelial proliferation and hypertrophy of the muscles of the gallbladder wall along with narrowing of distal bile duct which increases gallbladder intramural pressure leading to cystic dilatation of glands. Compartmentalization of gallbladder in segmental type AMH, results in exaggerated biliary stasis leading to increased cholelithiasis, which would explain the higher incidence of gallbladder carcinoma reported in this subtype [5]. The higher incidence of AMH in the older patient population [1] could also be explained on this basis. However, reports of cases of AMH in the pediatric population including rare cases in infancy raise concerns for an altogether different etiology underlying AMH [12,13].

None of the prior studies and reports on AMH focus on characterizing the spindle cell component in this entity. Most reports are based on the assumption that the spindle cell proliferation is predominantly smooth muscle representing the reactive smooth muscle hyperplasia in keeping with the proposed reactive etiology of this condition. Handra-Luca et al., in their study of AMH of the Vaterian system, briefly touch upon the predominantly myofibroblastic phenotype of most spindle cells, confirmed by a strong cytoplasmic expression of smooth muscle actin without desmin expression [14].

Myofibroblasts are ubiquitous cells that play significant roles in the ontogenesis, homeostasis, and tumorigenesis [24]. Though myofibroblasts have been shown to be present in several disease processes, their histogenesis remains unclear [25,26]. Myofibroblasts can be immunohistochemically distinguished from smooth muscle cells by their desmin negativity. In our study we found predominant myofibroblastic phenotype of the spindle cell component in all eighteen cases of AMH on assessment of H&E slides by light microscopy which was confirmed by performing a positive pixel count using digital imaging.

Myofibroblasts share the morphologic and functional characteristics of both fibroblasts and smooth muscle cells, and are thought to transdifferentiate from either one of these cell types [27]. Thus a plausible argument is that these myofibroblasts are indeed reactionary and on their way to becoming smooth muscle as a part of the hyperplastic response. However, the myofibroblastic population was significantly higher in AMH as compared to cholecystitis and chronic follicular cholecystitis in our study, waiving this argument and suggesting an etiology other than reactive nature of AMH suggested so far.

Myofibroblasts are the primary stromal element in polyps and cancer of the gastrointestinal tract and are responsible for the desmoplastic reaction character-

istic of colorectal carcinomas. The dominant presence of myofibroblasts in AMH, raises the suspicion of neoplastic nature of the glandular epithileum. The perineural invasion often reported in AMH is also suggestive of a neoplastic process [9]. This is further supported by the finding of significantly higher population of myofibroblasts seen in cases of invasive carcinoma of the gallbladder in our study. However, given the morphologically benign appearance of the glandular epithelium in AMH, along with the prior genetic studies, the glandular component in AMH appears to be non-neoplastic.

Another possible explanation for AMH could be diverticulosis of the gallbladder wall due to increased intramural pressure and subsequent induction of myofibrobastic proliferation being in response to diverticulitis or diverticular rupture. To test this hypotheisis, we assessed 10 cases of colonic diverticular disease, in various stages of evolution from diverticulosis to perforated diverticulitis, for myofibroblastic proliferation. None of the 10 cases in our study showed a significant myofibroblastic proliferation.

Given the significant myofibroblastic proliferation seen in AMH in our study we suggest primary stromal-epithelial derrangement to be the root cause of AMH. The myofibroblastic proliferation could be the factor causing local glandular obstruction as well as increased intramural pressure leading to cystic dilatation of the glands. Myofibroblasts secrete a variety of biochemical modulators, including chemotactic factors, signaling substances and cell receptor activators that may induce the migration of benign glands into nerve sheaths explaining the "perineural invasion" frequently seen in AMH [28]. A primary stromal epithelial derrangement would also explain the wide age distribution of AMH with rare cases being reported in infants and children. The rarity of these reports can be attributed to the fact that AMH is generally an incidental finding on gallbladder imaging and cholecystectomies, both of which are rarely required in the pediatric population. In the light of our study findings, the above discussion and the uncertain nature, we suggest the terms AMH and adenomyoma be dropped in favor of "adenomyofibromatosis", which is more accurate given the morphological and immunophenotypical nature of this lesion. Further characterization of this lesion as hamartomatous, developmental or neoplastic will require an extensive genetic workup of the myofibroblastic component.

Conclusions

In conclusion, despite the advent of immunohistochemistry and molecular studies, the exact mechanism of AMH has not been elaborated and its true nature in terms of neoplastic potential is still unclear. In this study we characterize the spindle cell component of this entity as predominantly myofibroblastic phenotype and suggest a primary stromal epithelial derangement as the basic etiopathogenesis of AMH.

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