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Mixed Endocrine–Exocrine Carcinoma of Gallbladder Derived from Dysplasia

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A rare case of multiple mixed endocrine-exocrine carcinoma (MEEC) of gallbladder in a 68-year-old man is described. The lesions were two separate nodules (17×13×7 mm and 17 mm in length) on the mucosa, which were composed of predominant neuroendocrine carcinoma (NEC) infiltrating into the adventitia and minor portion of adenocarcinoma (AC) or high grade dysplasia (HGD) on the surface. Surrounding mucosa showed areas of low grade dysplasia (LGD). Two nodal metastases out of 16 nodes were found containing NEC component. By immunohistochemistry, human mutL homolog 1 (hMLH1), p53, human mutS homolog 2 (hMSH2) and human mutS homolog 6 (hMSH6) showed diffuse strong positive reaction in HGD, AC and NEC, contrasting with weak positive reaction in LGD. On genetic analysis, all lesions of HGD, AC, and NEC except for LGD showed positive loss of heterozygosity in D5S346 locus. For microsatellite instability and *K-ras* mutation tests, all lesions showed negative results. Common immunophenotypes and molecular results among HGD, AC, and NEC suggested that NEC of this MEEC was derived from the dysplasia-AC sequence.

Key Words: Gallbladder; Carcinoma, neuroendocrine; Adenocarcinoma

Mixed endocrine-exocrine carcinoma (MEEC) of gastrointestinal tract has been known to be rare but in fact, a significant number of cases reported in older literature as carcinoids were MEECs. These tumors may display variable proportions of two components, with variable structural patterns, ranging from individually scattered neuroendocrine cells to well-formed neuroendocrine tumor with organoid nesting, trabecular, or solid growth patterns,[1,2] and variable grades of neuroendocrine component from carcinoid to well differentiated or poorly differentiated neuroendocrine carcinoma (NEC). Although these variations show organ dependent pattern in general, clear definitions and diagnostic features are still missing, as well as a definite knowledge of their biological properties and histogenesis.

In gastrointestinal tract, MEEC was defined as epithelial malignant tumors characterized by a combination of a predominant exocrine component and a neuroendocrine cell subpopulation more than one third of total tumor volume.[1] In the pancreas, MEEC includes mixed ductal-endocrine and mixed acinar-endocrine carcinoma. However, MEEC of gallbladder is very rare and only a few cases have been reported so far as adenoendocrine cell carcinoma of gallbladder.[3-5]

It has been well established that invasive gallbladder carcinoma is preceded by preneoplastic lesions, including dysplasia and carcinoma *in situ* (CIS). In addition, metaplasia of intestinal or pyloric type is frequently observed near carcinomas, but its malignant potential is unknown.[6] The genetic alterations commonly observed in sporadic biliary tract cancers include mutations of the *K-ras, p16,* adenomatosis polyposis coli, *β-catenin,* and *p53* genes, p53 overexpression, and loss of heterozygosity (LOH) of chromosomal arms.[7] Microsatellite instability (MSI) status in gallbladder cancer is different according to the histologic subtype and geographical and racial variants.[8]

MEEC of gallbladder with dominant NEC with the remaining mucosa showing various spectrum of mucosal change from low grade dysplasia (LGD) to high grade dysplasia (HGD) is described in the article. The results of the immunohistochemical and genetic analysis including MSI, LOH, and *K-ras* mutation suggest that NEC might be derived from dysplasia-adenocarcinoma (AC) sequence.

CASE REPORT

A 68-year-old man was admitted the hospital with a polypoid mass in gallbladder which was detected in regular checkup. He had no previous history or family history. On laboratory examinations, there was no abnormal finding. No clinical sign of tumor hormonal activity was observed. On abdominal ultra-

sonogram, a polypoid mass, measuring 17 mm in the greatest diameter, was detected in the body of gallbladder. The mass was single, fixed, and hypoechoic with echogenic rim (Fig. 1A). Cholecystectomy was performed. In cholecystectomy specimen, the mucosa showed an ovoid nodular mass in the body, measuring 17×13×7 mm in size. The cut surface was homogeneously whitish tan, solid and infiltrated the adventitia (Fig. 1B). There was another separate plaque-like elevated lesion, measuring 17 mm in greatest diameter, 10 mm apart from the above mass (Fig. 1B).

On microscopic examination, the nodular mass consisted of two distinct tumor components with narrow transitional area. The major tumor part was made by nodular growing mass showing solid sheets of densely packed, severe atypical cells with high N/C ratio (Fig. 2A). The nuclei were ovoid with finely granular chromatin and inconspicuous nucleoli (Fig. 2C). The tumor margin was infiltrative to the adventitia. By immunostaining, these tumor cells were stained intensely and uniformly for CD56 (1:100, Novocastra, London, UK) (Fig. 2B). These features were consistent with a diagnosis of high grade NEC. On the surface of this mass, there was second pattern of neoplastic glands occupying only a small portion of the mass, measuring 3 mm in width (Fig. 2A). They were irregular in shape and lined by atypical cells, consistent with well differentiated AC. It infiltrated focally and superficially the lamina propria of mass, but was limited in mucosa (Fig. 2A). Some of glands were lined by NEC cells forming transition zones (Fig. 2D). The second lesion was disclosed as another MEEC, which was a mixture of HGD, CIS, and NEC (Fig. 2E, F). The HGD and CIS grew forming plaque-like elevated lesion (Fig. 2F) and NEC component showed infiltrative growth to adventita (Fig. 2E). There was an area of mixed zones of CD56-positive proliferative NEC cells arising from or standing abreast the entrapped glands (Fig. 2G, H). The remaining mucosa showed low grade dysplastic change, and a few metaplastic cells were associated only with

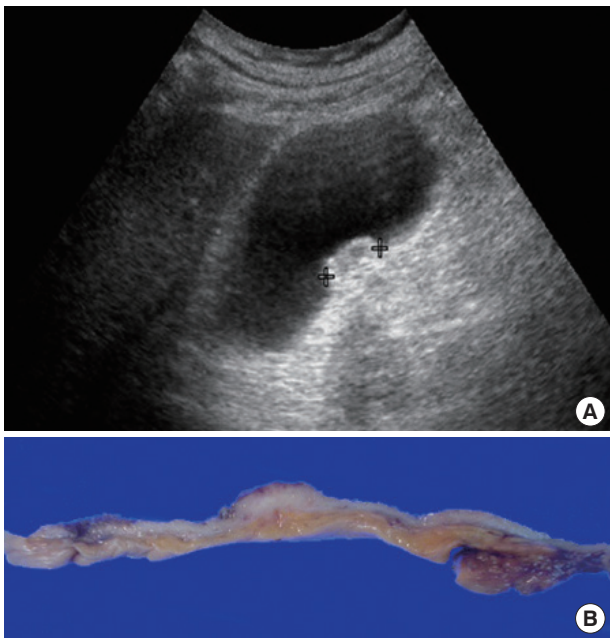

Fig. 1. Ultrasonogram shows a hypoechoic mucosal lesion with echogenic rim, measuring 17 mm in diameter (A). In cholecystectomy specimen, the cut surface reveals a whitish tan solid mass in the body and another plaque-like lesion, 10 mm apart from the nodular mass (B).

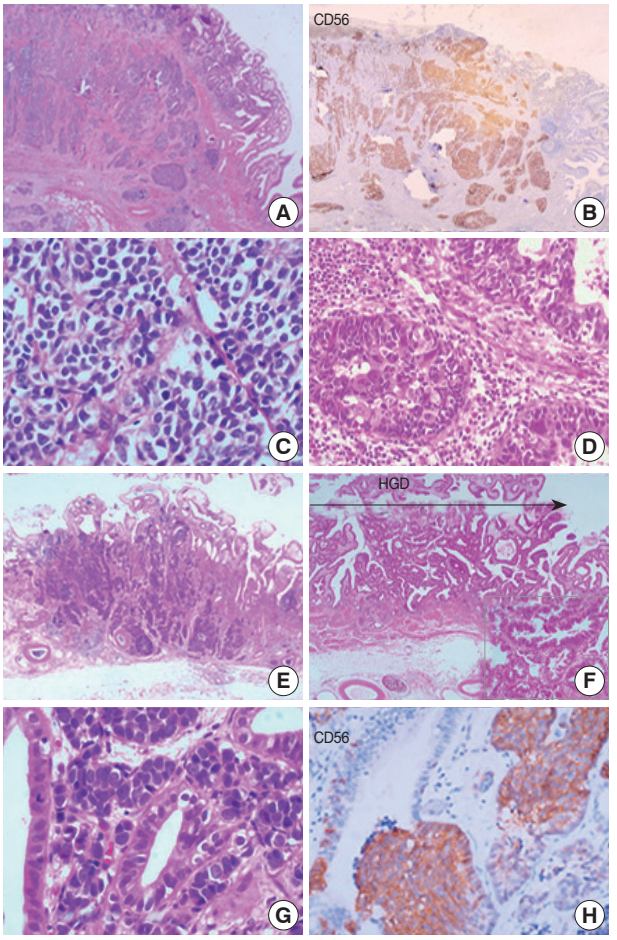

Fig. 2. The majority of the mass is neuroendocrine carcinoma (NEC), infiltrating the subserosa (A). NEC cells are homogeneously round with high N/C ratio (C) and show diffuse positive reaction in CD56 immunostaining (B). A small portion of adenocarcinoma (AC) is well differentiated and limited in mucosa (A). There is the transition zone between AC and NEC (D). In another lesion, the high grade dysplasia (HGD) and carcinoma *in situ* form a plaque-like elevated lesion and the NEC component shows infiltrative growth to subserosa (E, F). Intimate relations between NEC and AC are observed (G, H).

LGD but not with NEC. The tumor was diagnosed as MEEC accompanied by a spectrum of mucosal change from LGD to HGD in the remaining mucosa.

Additional immunostaining results for p53 (1:100, Novocastra), human mutL homolog 1 (hMLH1; 1:100, Zymed, San Francisco, CA, USA), human mutS homolog 2 (hMSH2; 1:100, Zymed) and human mutS homolog 6 (hMSH6; 1:100, Zymed) were graded as 4 categories, 0, 1+, 2+, and 3+, according to its intensity and positive cell proportion. The intensity was classified as 0, 1+, 2+, and 3+, and positive cell proportion as 0, 1: <1/10, 2: 1/10-1/3, 3: 1/3-2/3, and 4: >2/3. According to the sum of the intensity and positive cell proportion, it is classified as negative: 0-2, 1+: 3, 2+: 4-5, and 3+: 6-7. p53 showed diffuse strong positive reaction (3+ or >3+) in HGD, AC, and NEC (Fig. 3). LGD showed 2+ positivity. hMLH1 showed positive reaction in AC and NEC. In HGD, focal positive reaction was seen and in LGD, trace (Fig. 3). hMSH2 showed strong positive reaction (2-3+) in HGD, AC, and NEC, whereas weak positive reaction in LGD (Fig. 3). hMSH6 showed also diffuse strong positive reaction (3+ or >3+) in HGD, AC, and NEC. LGD showed 2+ positivity (Fig. 3).

Each portion of the tumor was tested for MSI and *K-ras* mutation. MSI status was assessed by examining five independent genomic sites, including two mononucleotide repeat microsatellites (BAT25 and BAT26) and three dinucleotide repeat microsatellites (D2S123, D5S346, and D17S250) as recommended by the National Cancer Institute Workshop.[9] The sample was considered to show MSI if 2 or more of the markers showed loss of heterozygosity. All four lesions of LGD, HGD, AC, and NEC showed negative MSI but HGD, AC, and NEC showed LOH in D5S346 locus (Fig. 4). *K-ras* mutation was assessed by direct sequencing using the ABI-PRISM BigDye Terminator ver. 3.1 (Applied Biosystems, Foster, CA, USA) with both forward and reverse sequence-specific primers. All four lesions of LGD, HGD, AC, and NEC showed no *K-ras* mutation (Fig. 4). Immunohistochemical and molecular results are summarized in Table 1.

The tumor stage was T1N1M0 with two lymph nodes with metastatic NEC. The patient did not receive any adjuvant che-

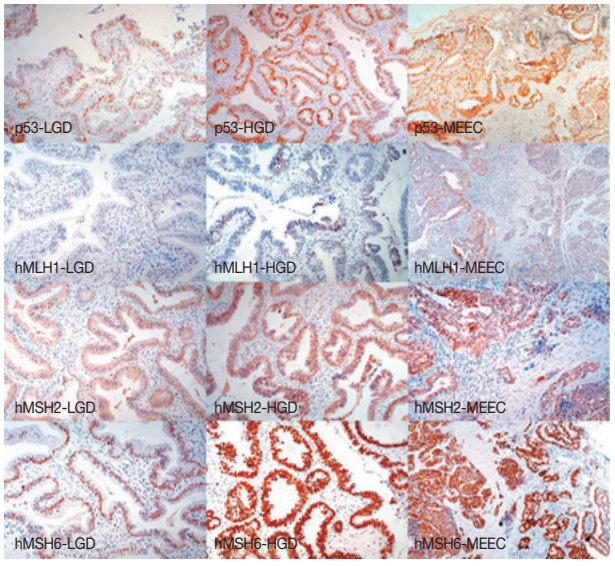

Fig. 3. Immunohistochemistry of p53, human mutL homolog 1 (hMLH1), human mutS homolog 2 (hMSH2), and human mutS homolog 6 (hMSH6) in low grade dysplasia (LGD), high grade dysplasia (HGD), and mixed exocrine-endocrine carcinoma (MEEC). The overexpression patterns are remarkably distinguished between LGD and higher dysplastic group including HGD, adenocarcinoma, and neuroendocrine carcinoma.

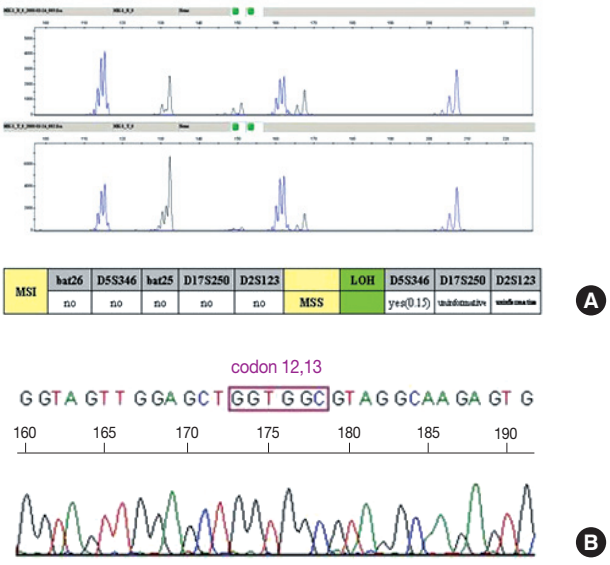

Fig. 4. Neuroendocrine carcinoma, adenocarcinoma, and high grade dysplasia are microsatellite instability (MSI) negative in all five markers with loss of heterozygosity (LOH) in D5S346 locus, and show no *K-ras* mutation. MSS, microsatellite stable.

Table 1. Results of immunohistochemistry and genetic analysis

	IHC				MSI	LOH	*K-ras* mutation
	p53	hMLH1	hMSH2	hMSH6			
LGD	2+	+/-	1+, weak	2+	MSS	N	n
HGD	3+	1+	2+	3+	MSS	D5S346	n
AC	3+	3+	3+	3+	MSS	D5S346	n
NEC	3+	2+	3+	3+	MSS	D5S346	n

Negative, 0-2; 1+, 3-4; 2+, 5; 3+, 6-7.
IHC, immunohistochemistry; hMLH1, human mutL homolog 1; hMSH2, human mutS homolog 2; hMSH6, human mutS homolog 6; MSI, microsatellite instability; LOH, loss of heterozygosity; LGD, low grade dysplasia; MSS, microsatellite stable; N, negative; n, no mutation; HGD, high grade dysplasia; AC, adenocarcinoma; NEC, neuroendocrine carcinoma.

motherapy and has shown no evidence of disease for 12 months after the surgery.

DISCUSSION

The origin of NEC of gallbladder has not been thoroughly investigated yet. In older literatures about the origin of gallbladder carcinoid tumors, the author proposed that gallbladder carcinoid tumors might develop from endocrine cells induced by intestinal metaplasia of the body and fundus.[10] However, in this case, the NEC was not associated with intestinal metaplasia but with dysplastic change of mucosa, especially with HGD. NEC was combined with not only AC but also HGD, forming two different masses. The LGD showed no association with NEC and only with a few metaplastic cells. Another interesting feature of this case was that the major component of the main mass was NEC with minute surface AC component in spite of this sequential mucosal change in the remaining mucosa. And the NEC infiltrated to subserosa and metastasized to lymph nodes. According to the literature, the NEC easily metastasize to other organ in spite of their small size.[11] However, the mixture of dominant NEC with minute AC like this case is very rare, especially in gallbladder.

The precise molecular mechanisms of development and progression of the ominous gallbladder carcinoma is still unclear, but recently, a high incidence of LOH at several chromosomes in gallbladder carcinoma has been found. Wistuba *et al.*[6] presented that LOH at *p53* (91%), 9p (50%), 8p (44%) and deleted in colorectal carcinoma (DCC) (31%) were frequent early events, while LOH at 3p, rb, and 5q occurred occasionally in gallbladder carcinoma. In particular, LOH on chromosomes 1p, 3p, 5p, 8p, 9p, 9q, 13q, 16q, and 17p has been highlighted and LOH on 3p, 9p, 13q, and 17p has been detected in preneoplastic lesions and in the early phase of gallbladder carcinoma during multistep carcinogenesis.[12] According to the previous report, LOH at *p53* occurred more frequently and earlier than protein overexpression.[6] In this case, LOH at 5q region (D5S346) was found in HGD, AC, and NEC, except for LGD. The immunostaining results also distinguished between LGD and higher dysplastic group including HGD, AC, and NEC. These results strongly suggested that NEC might be derived from the sequential change of HGD-AC sequence.

Little is known about the incidence of MSI-high group and underlying DNA mismatch repair (MMR) defects in gallbladder carcinogenesis. Sessa *et al.*[13] analyzed MSI status from a large series of gallbladder carcinomas to clarify the role of MSI attributable to defective DNA MMR in these tumors.[12] Their results indicated that none of the 71 gallbladder cancers analyzed exhibited MSI and loss of immunohistochemical expression for either hMLH1 or hMSH2 proteins.[13] In our case, all four lesions of LGD, HGD, AC, and NEC showed no MSI in all five markers and showed immunohistochemical expressions for p53, hMLH1, hMSH2, and hMSH6. From these results, a defective MMR system does not seem to be involved in this gallbladder carcinogenesis.

According to previous report, *K-ras* mutation was present in 15.2% of bile duct and 2.7% of gallbladder cancers.[7] Lesions that are precursors of invasive carcinomas of the biliary tract show differences in *K-ras* mutations in different subsites. The frequency of *K-ras* mutation is also dependent on the racial or geographical variations.[14-16] Dysplasia and CIS of gallbladder, the precursors of invasive gallbladder carcinomas, lack *K-ras* mutations,[17] but gallbladder adenomas, which are not considered precursors of invasive gallbladder carcinoma, do have *K-ras* mutations.[7,18] In our case, all four lesions of LGD, HGD, AC, and NEC showed no *K-ras* mutation. It might be suggested that this MEEC was developed by a *K-ras* independent pathway.

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