TITLE PAGE

Manuscript Title: Resection of Lung metastasis from Gallbladder Carcinoma: Immunohistochemistry of RCAS1 and $CD8^+$ T cells in Primary and Metastatic Tumors

Authors and their affiliations: Taro Oshikiri, MD[1], Takayuki Morita, MD[1], Miyoshi Fujita, MD[1], Yuji Miyasaka, MD[1], Naoto Senmaru, MD[1], Hidehisa Yamada, MD[1], Naoto Kaji, MD[2], and, Satoshi Kondo, MD[2]

Department of Surgery, Hokkaido Gastroenterology Hospital, Honcho 1jo, 1chome, Higashi-ku, Sapporo, Hokkaido, 065-0014, Japan

2. Department of Surgical Oncology, Cancer Medicine, Division of Cancer Medicine, Hokkaido University Graduate School of Medicine, Kita-15, Nishi-7, Kita-ku, Sapporo, Hokkaido, 060-8648, Japan

Corresponding and reprint request to: Taro Oshikiri, MD

Department of Surgery, Hokkaido Gastroenterology Hospital, Honcho 1jo, 1chome, Higashi-ku, Sapporo, 065-0014,Sapporo, Japan

Telephone number: 81-11-784-1811 (200)

Fax number: 81- 11-784-1838

E-mail address: tarotaro@yf7.so-net.ne.jp

Information: We wish the paper to be published in Tumor Biology.

Key Words: immunohistochemistry, $CD8^+$ T cells, thyroid transcription factor 1, RCAS1

Abstract

Advanced Gallbladder cancer has an extremely poor prognosis. We examined a patient with resectable gallbladder cancer with associated lung metastasis. A 64-year-old female patient, diagnosed with gallbladder cancer and a solitary benign lung tumor by imaging, was subjected to extensive cholecystectomy and extrahepatic bile duct resection. After one year, a follow-up CT indicated enlargement of the lung tumor; video-assisted right middle lobectomy was then performed. The lung tumor was diagnosed as a metastasis derived from the gallbladder cancer by pathology and immunohistochemistry. Expression of RCAS1, an independent unfavorable prognostic indicator in gallbladder cancer, was observed in both the gallbladder and lung tumors. However, infiltration of $CD8^+$ T cells was only seen in the lung metastatic tumor. She has remained free of any evidence of recurrence in the 10 months and 4 years after the first surgery. The results that metastasis is solitary and infiltrated by $CD8^+$ T cells correspond with the present clinical history.

Introduction

While gallbladder cancer (GBC) has an extremely poor prognosis, recent advances introducing extended surgical therapy combining liver resection with wide lymph node dissection is beginning to improve the long-term survival of patients with GBC [1, 2]. Using this methodology, Tsukada *et al.* achieved excellent 5-year survival rates in patients with stage I and II tumors. The 5-year survival rate, however, is highly reduced to less than

5% when noncurative resections involving distant metastasis were performed [3]. To understand the molecular basis of GBC, we previously investigated a novel tumor-associated antigen found in GBC; Receptor-binding cancer antigen expressed on SiSo cells (RCAS1) correlated significantly with tumor progression, predicting a poor outcome in gallbladder cancer [4]. In this study, we examined a rare case of long-term disease-free survival in a patient with a solitary metastatic lung tumor derived from GBC. We identified the lung tumor as a metastasis by immunostaining with anti-thyroid transcription factor 1 (TTF-1) antibodies. To investigate the characteristics of the primary gallbladder and metastatic lung cancer cells, we also examined the expression of RCAS1 and infiltration of $CD8^+$ T cells.

Case report

In March of 2000, a 64-year-old female patient was referred to our hospital with right upper quadrant pain. Laboratory findings were unremarkable, with negative results for carcinoembryonic antigen (CEA). Magnetic resonance cholangiopancreatography (MRCP) and computed tomography (CT) of the abdomen revealed a gallbladder tumor (Figure 1A, B). CT of the lung indicated a solitary tumor in the right middle lobe. As neither spicula nor pleural effusions were seen, we diagnosed the tumor as benign (Figure 2A). We performed extended cholecystectomy and extrahepatic bile duct resection with lymph node resection on March 28, 2000. The pathological diagnosis was gallbladder carcinoma. One year after

the surgical procedure, a follow-up CT of the lung demonstrated an enlargement of the lung tumor without any additional lesions (Figure 2B). With diagnoses of primary lung cancer or solitary lung metastasis from the primary gallbladder carcinoma, we performed video-assisted right middle lobectomy on April 23, 2001. The patient has remained free of any evidence of recurrence or additional metastasis in the 10 months and 4 years after the first surgery.

Pathological Examination

Macroscopically, we discovered a soft papillary tumor within the body of the gallbladder (Figure 3A). In the specimen from the right lung, we observed a white nodular tumor (Figure 3B). Upon microscopic examination, the papillary adenocarcinoma appeared to infiltrate the gallbladder sub-serosa. Vascular or lymphatic tumor involvement was apparent, but no lymph node metastasis could be observed (Figure 4A). In the right lung specimen, the adenocarcinoma, appearing similar to the primary gallbladder tumor, demonstrated significant necrosis (Figure 4B).

Methods

Immunohistochemistry

Paraffin-embedded, formalin-fixed tissue blocks were sectioned at 4 μm, heated at 60^{o}C for 30 minutes, deparaffinized, and hydrated using a xylene and alcohol series.

Optimum pretreatment and dilutions were determined by testing known positive and negative control materials. We utilized the following mouse monoclonal primary antibodies for staining: anti-human TTF-1 (Dako; clone 8G7G3-1; dilution 1:150), anti-human CD8 (Dako; clone C8/144b; dilution 1: 100), and anti-human 22-1-1 (Medical & Biological Laboratories, Tokyo, Japan; dilution 1:500).

Evaluation of immunostaining

For TTF-1, tumors demonstrating cytoplasmic immunoreactivity were selected for further analysis by grading the intensity of the stain. Tumors were characterized as 3+ when positive staining could be observed upon examination of the slide with the 4 objective, 2+ when positivity was seen using the 10 objective, 1+ when staining could be seen only at a magnification of 40, and 0 if no staining could be observed even when using the 40 objective. Samples were also divided into categories based on the percentage of positive cells, with groups of more than 50% positive tumor cells, between 10% and 50%, less than 10%, and no cells [5].

The number of immunoreactive $CD8^+$ T cells detected within the cancer cell nests were quantified with a microscopic field of ´200 (0.933 mm^2). Five areas with most abundant infiltration were selected, and the average numbers of 0, 1-9, 10-29, and over 30 were scored as group 0, I, II, and III, respectively. Group I, II, and III were considered positive patterns, while group 0 was considered negative [6].

The evaluation of the degree of RCAS1 immunoreactivity was performed as described by Oshikiri *et al*. [4], classifying cases into four categories. In RCAS1 I cases, fewer than 25% of tumor cells demonstrated immunoreactivity; in RCAS1 II, 25% to 50% of the tumor cells exhibited staining; in RCAS1 III, 50% to 75% were positive; and in RCAS1 IV, more than 75% expressed the tumor antigen. RCAS1 II, III, and IV group tumors were considered to be high-expression samples, while RCAS1 I group tumors were low-expression samples [4].

Results

Immunostaining for TTF-1 was not observed in either the gallbladder or lung carcinoma cells; both tumors demonstrated staining intensities of 0 and staining percentages less than 10%. (Figure 5A, B). Positive RCAS1staining, however, was observed in both the gallbladder (RCAS1 III) and lung (RCAS1 IV) carcinoma cells (Figure 5C, D).

Concerning $CD8^+$ T cell, gallbladder tumor was scored as 0 (negative), while lung tumor was scored as III (positive) (Figure 5E, F). The distribution of cells expressing RCAS1 differed from the pattern of $CD8^+$ T cell infiltration in the same sections of the lung tumor.

Discussion

TTF-1, a member of the Nkx2 family of homeodomain-containing transcription

factors, is expressed in the lung epithelium. This marker is used to distinguish tumors of pulmonary origin within other body sites. Positive TTF-1 immunostaining was seen in 74% of 42 primary lung adenocarcinomas [7]. As TTF-1 appears to be a highly specific marker of primary lung adenocarcinomas, TTF-1 expression should be examined for the differential diagnosis of primary and metastatic adenocarcinomas in the lung [8]. The expression profiles of these markers suggest that the lung tumor in our patient is not a primary tumor, but rather a metastasis derived from the primary gallbladder cancer.

A cDNA encoding the antigen recognized by the 22-1-1 antibody was designated the receptor-binding cancer antigen expressed on SiSo cells (RCAS1). It was initially proposed that the RCAS1 gene product is an integral membrane protein functioning as a ligand for a putative receptor present on normal peripheral lymphocytes, including T, B, and natural killer cells. Tumor cells may evade immune surveillance via expression of RCAS1, which may suppress clonal expansion and induce apoptosis in these immune cells possessing RCAS1 receptors [9]. In this study, positive RCAS1 staining was seen in the primary gallbladder tumor (RCAS1 III). We previously reported that strong RCAS1 expression by GBC significantly associated with venous involvement [4], consistent with the hematogenous lung metastasis seen in this case. Interestingly, higher expression of RCAS1 was seen in the metastatic lung tumor (RCAS1 IV) than in the primary tumor. We observed a similar phenomenon in colorectal cancers with metastases to the liver. In eight of nine patients, RCAS1 expression was higher in the secondary liver tumors than in the primary

tumors (data not shown). Cancer cells expressing RCAS1 may selectively travel to the lung, creating a metastatic focus in which all the cells express high levels of RCAS1. Our previous report suggested that high RCAS1 expression in GBC is an independent unfavorable prognostic factor. GBC expressing RCAS1 may have a higher potential to produce hematogenous metastases, leading to a poor prognosis and probably fatality. Some exceptions, including our patient, have been reported. Elias *et al*. reported long survival cases of resectable liver metastasis from GBC [10] in which RCAS1 was one of the important prognostic factors. Suzuki et al. resected lung metastasis from GBC by thoracospopic surgery [11]. There may be additional factors that may aid in the effective selection of operative cases with GBC metastasis.

In our case, no evidence of infiltration of $CD8^+$ T cells was seen in primary GBC tumor. However, massive infiltration of $CD8^+$ T cells was seen in metastatic lung tumor. Tumor-infiltrating lymphocytes, particularly $CD8^+$ T cells, may be of the hallmark of productive antitumor immunity and specifically recognize tumor cell epitopes [6]. If RCAS1 exhibits an immunosuppressive function, it is difficult to reconcile the observation that high levels of $CD8^+$ T cell infiltration are observed in metastatic lung tumors demonstrating strong expression of RCAS1. Thus, the molecular function of RCAS1 in humans remains controversial. Recently, Engelsberg et al. reported that RCAS1 modulates the surface expression of a normally cryptic tumor-associated *O*-linked glycan structure containing *N*-acetyl-D-galactosamine, GalNAc (Tn). The 22-1-1 mAb failed to recognize

RCAS1, instead recognizing Tn, because the 22-1-1 epitope recognized by this antibody is identical to the Tn structure. This research demonstrated that the mAb 22-1-1 recognizes RCAS1 indirectly through a Tn modification [12]. Tn antigens, which are modulated by RCAS1, are generally involved in cell adhesion, invasion, and metastasis of cancer cells [13]. The immunosuppressive function of the Tn antigen, however, is unclear. Our immunohistochemical data could not identify a correlation between regions of RCAS1 expression and sites of $CD8^+$ T cell infiltration. Thus, little evidence exists to suggest an interaction between RCAS1 and $CD8^+$ T cells. The differences in $CD8^+$ T cells status between a primary tumor of the gallbladder and a metastatic recurrence in the lung may be dependent on organ specificity, not RCAS1 expression. In the lung, the degree of $CD8^+$ T cell infiltration into cancer cell nests or mesenchymal stroma is higher than that seen in the primary gall bladder tumor. While infiltrating $CD8^+$ T cells are a positive prognostic independent factor in GBC, these cells do not appear to function as effectors in lung adenocarcinomas [14, 15]. The exceptional status of the cancer cells, which originated from gallbladder and were located within the lung, is unique to our patient. $CD8^+$ T cells infiltrated and suppressed the metastatic cancer cells derived from the gall bladder present in the lung, such that multiple metastases or new metastatses may be exempted. In general, operative indication is not seen in metastatic foci in patients with GBC. Nevertheless, a long-term solitary metastasis which is infiltrated by $CD8^+$ T cells can occasionally be effectively treated by surgical resection. It will be important in the future to determine the

other prognostic factor predicting the efficacy of such treatment.

Conclusion

We observed the long-term disease-free survival of a patient with GBC, in whom both the primary GBC and a solitary metastatic lung tumor expressed high levels of RCAS1. The results that resected metastasis is solitary and infiltrated by $CD8^+$ T cells correspond with the present clinical history.

References

1 Shimada H, Endo I, Togo S, Nakano A, Izumi T, Nakagawara G, The role of lymph node dissection in the treatment of gallbladder carcinoma, Cancer 79 *(*1997) 892-899.

2 Kondo S, Nimura Y, Hayakawa N, Kamiya J, Nagino M, Uesaka K, Extensive surgery

for carcinoma of the gallbladder, Br J Surg 89 (2002) 179-184.

3 Tsukada K, Hatakeyama K, Kurosaki I, Uchida K, Shirai Y, Muto T, Yoshida K, Outcome of radical surgery for carcinoma of the gallbladder according to the TNM stage, Surgery 120 (1996) 816-821.

4 Oshikiri T, Hida Y, Miyamoto M, Hashida H, Katoh K, Suzuoki M, Nakakubo Y, Hiraoka K, Shinohara T, Itoh T, Kondo S, Katoh H, RCAS1 as a tumour progression marker: an independent negative prognostic factor in gallbladder cancer, Br J Cancer 84 (2001) 1922-1927.

5 Bejarano PA, Mousavi F, Incidence and significance of cytoplasmic thyroid transcription factor-1 immunoreactivity, Arch Pathol Lab Med 127 (2003) 193-195.

6 Oshikiri T, Miyamoto M, Shichinohe T, Suzuoki M, Hiraoka K, Nakakubo Y, Shinohara T, Itoh T, Kondo S, Katoh H, Prognostic value of intratumoral CD8+ T lymphocyte in extrahepatic bile duct carcinoma as essential immune response, J Surg Oncol 84 (2003) 224-228.

7 Zou SM, Lin DM, Lu N, Liu XY, Wen P, Liu FS, Use of thyroid transcription factor-1, surfacfant protein-B, cytokeratin 7 and cytokeratin 20 in discrimination between primary and metastatic adenocarcinoma of lung, Zhonghua Yi Xue Za Zhi 83 (2003) 1350-1352. Chinese.

8 Reis-Filho JS, Carrilho C, Valenti C, Leitao D, Ribeiro CA, Ribeiro SG, Schmitt FC, Is TTF1 a good immunohistochemical marker to distinguish primary from metastatic lung

adenocarcinomas? Pathol Res Pract 196 (2000) 835-840.

9 Nakashima M, Sonoda K, Watanabe T, Inhibition of cell growth and induction of apoptotic cell death by the human tumor-associated antigen RCAS1, Nat Med 5 (1999) 938-942.

10 Elias D, Cavalcanti de Albuquerque A, Eggenspieler P, Plaud B, Ducreux M, Spielmann M, Theodore C, Bonvalot S, Lasser P, Resection of liver metastases from a noncolorectal primary: indications and results based on 147 monocentric patients, J Am Coll Surg 187 (1998) 487-493.

11 Suzuki I, Oho K, Tamura K, Ohtani Y, Tanaka Y, Serizawa H, Pulmonary resection for metastatic gallbladder carcinoma by thoracoscopic surgery--report of a case, Nippon Kyobu Geka Gakkai Zasshi 42 (1994) 607-610. (In Japanese with English abstract)

12 Engelsberg A, Hermosilla R, Karsten U, Schulein R, Dorken B, Rehm A. The Golgi protein RCAS1 controls cell surface expression of tumor-associated O-linked glycan antigens, J Biol Chem 278 (2003) 22998-23007.

13 Cao Y, Karsten UR, Liebrich W, Haensch W, Springer GF, Schlag PM, Expression of Thomsen-Friedenreich-related antigens in primary and metastatic colorectal carcinomas. A reevaluation, Cancer 76 (1995) 1700-1708.

14 Wakabayashi O, Yamazaki K, Oizumi S, Hommura F, Kinoshita I, Ogura S, Dosaka-Akita H, Nishimura M, CD4+ T cells in cancer stroma, not CD8+ T cells in cancer cell nests, are associated with favorable prognosis in human non-small cell lung cancers,

Cancer Sci 94（2003）1003-1009.

15 Nakakubo Y, Miyamoto M, Cho Y, Hida Y, Oshikiri T, Suzuoki M, Hiraoka K, Itoh T, Kondo S, Katoh H, Clinical significance of immune cell infiltration within gallbladder cancer, Br J Cancer 89（2003）1736-1742.

Figure legends

Figure 1.

(A) CT of the abdomen and (B) magnetic resonance cholangiopancreatography (MRCP) revealed the presence of a gallbladder tumor (arrows).

Figure 2.

（A）CT of the lung exhibited a solitary tumor（arrow）in the right middle lobe upon first admission.（B）A solitary enlarged lung tumor（arrow）was observed in the right middle lobe one year after initial surgery.

Figure 3.

（A）Macroscopically, a soft papillary tumor was found within the body of the gallbladder.

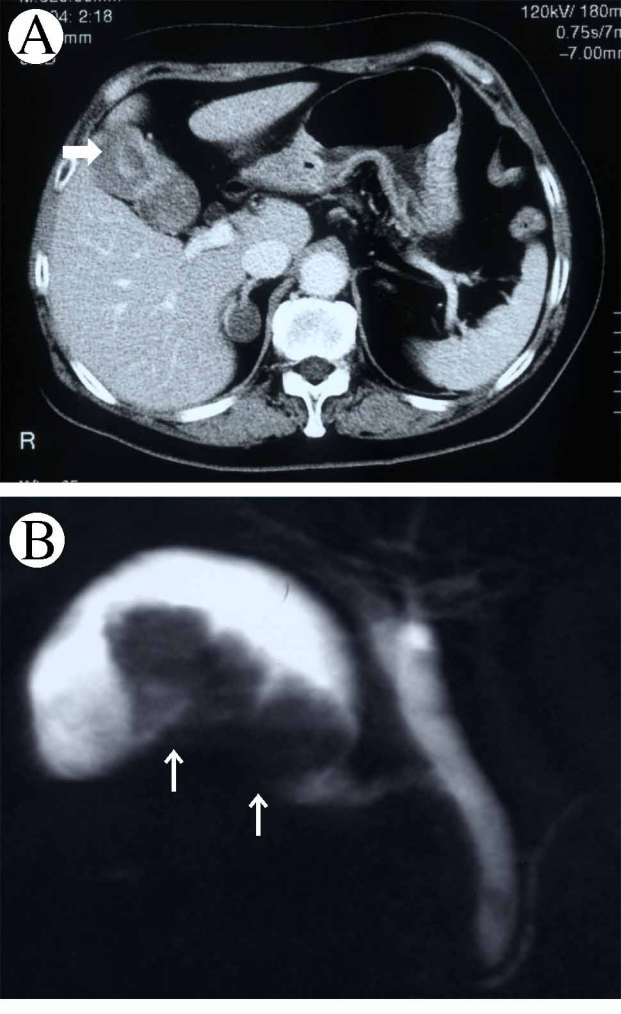
（B）In the right lung specimen, we observed a white nodular tumor.

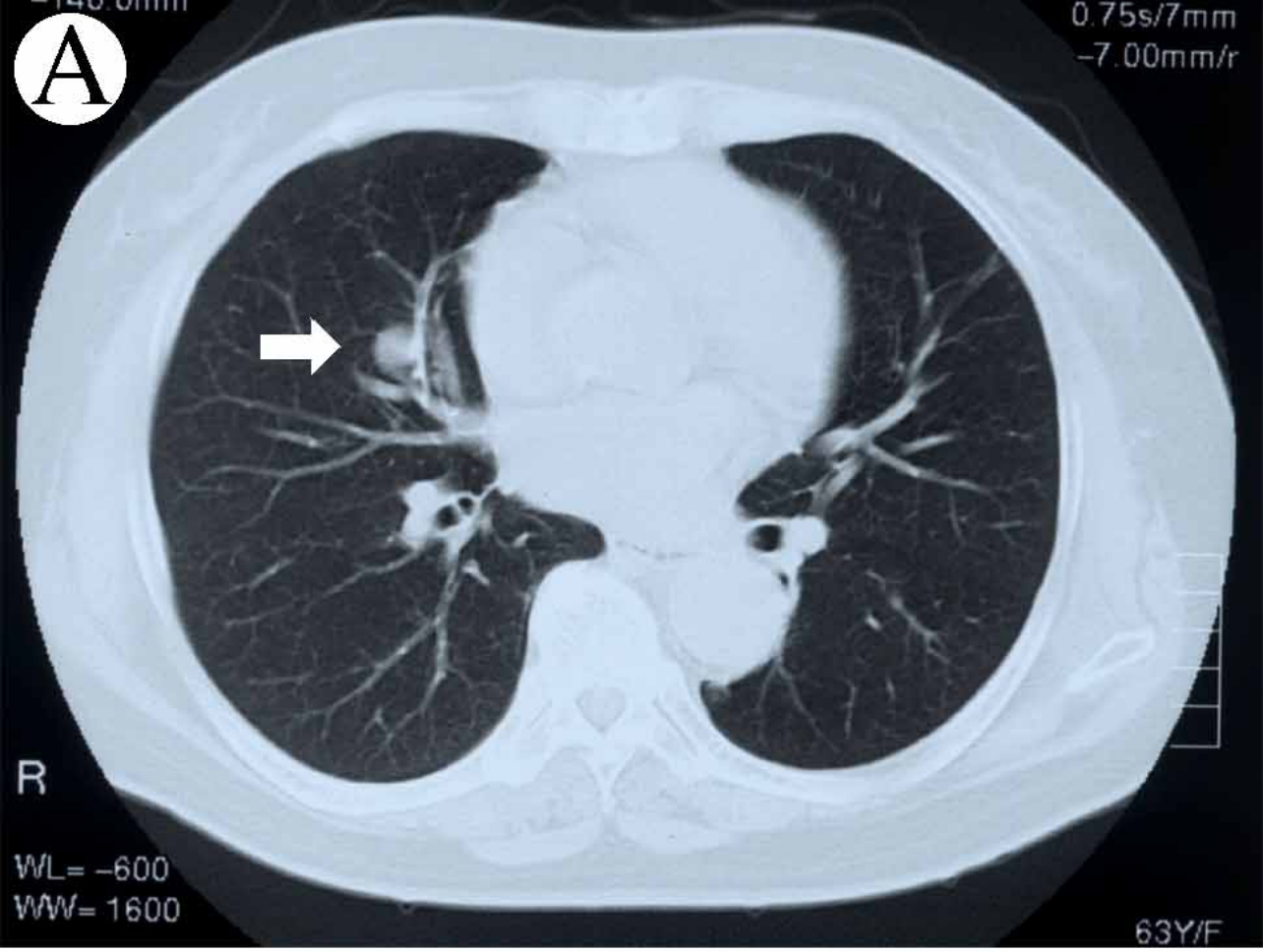
Figure 4.

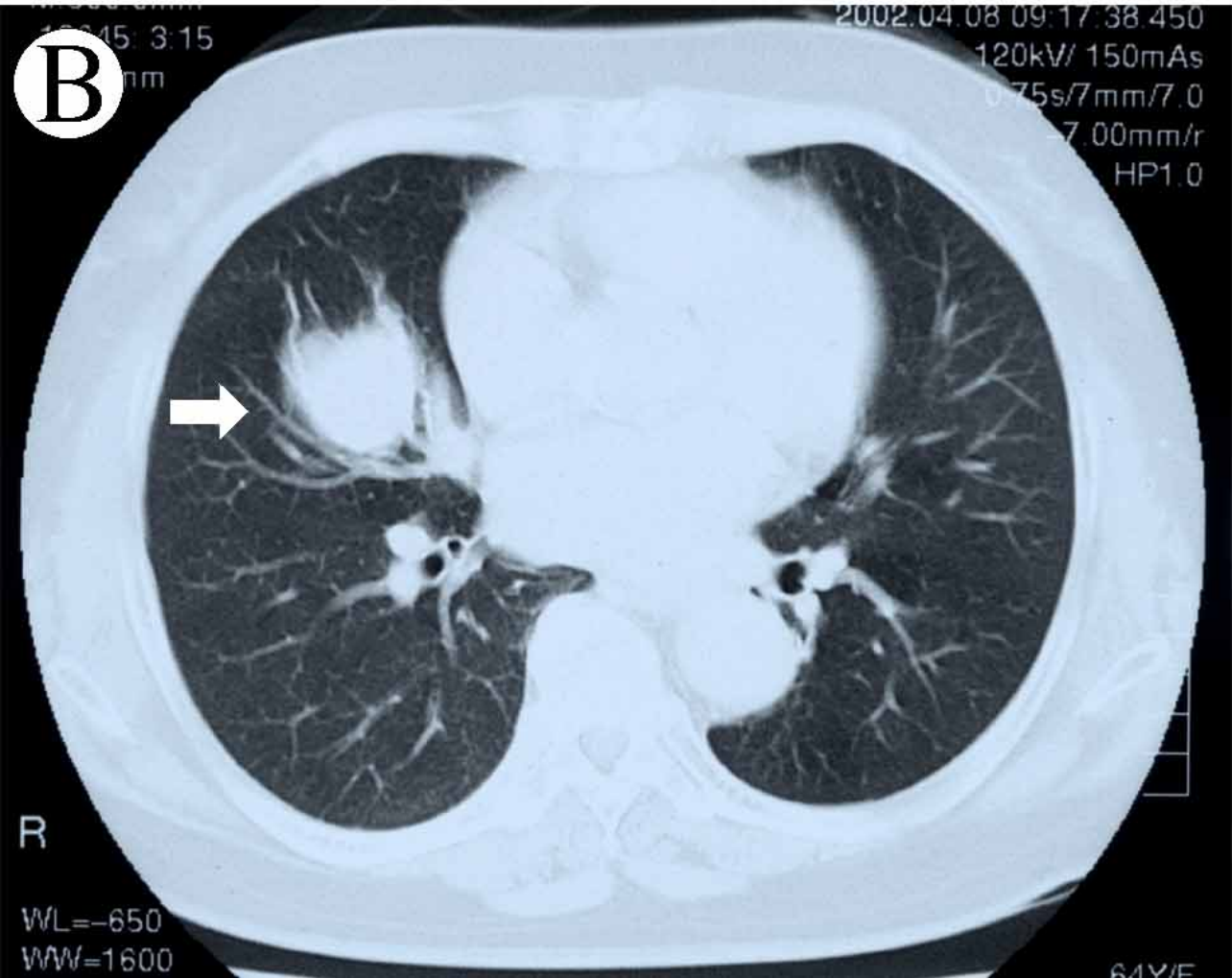
(A) Upon microscopic examination, we observed the infiltration of a papillary adenocarcinoma into the gallbladder sub-serosa. (B) This adenocarcinoma was similar to the gallbladder tumor; significant necrosis was also seen in the lung specimen. Sections were stained with hematoxylin and eosin (x100).

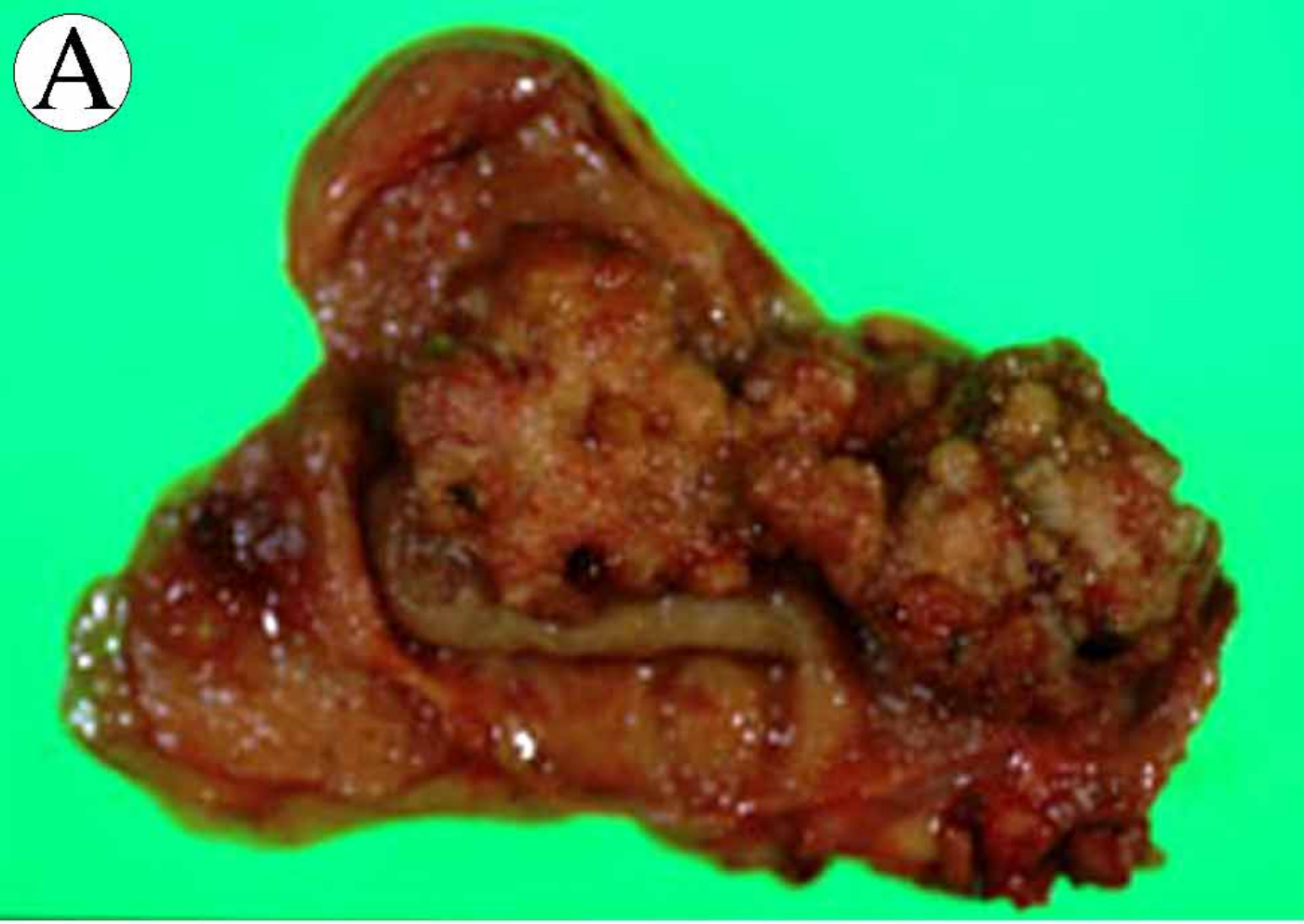
Figure 5.

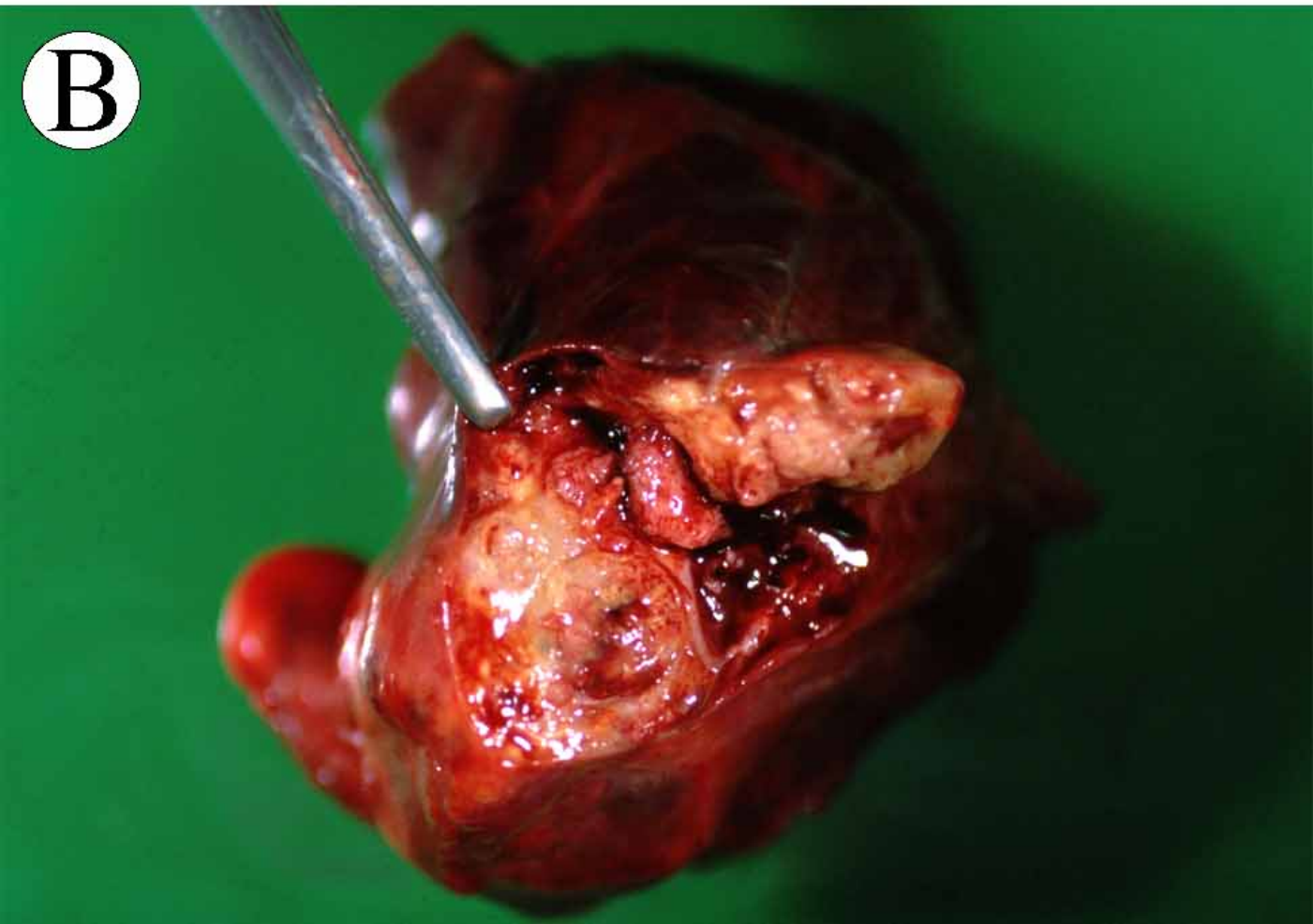
TTF-1 immunostaining was negative for both (A) the gallbladder and (B) lung carcinoma cells. RCAS1 expression was observed in (C) the gallbladder carcinoma cells (RCAS1 III). (D) The lung carcinoma cells demonstrated stronger expression of RCAS1 (RCAS1 IV) than the gallbladder carcinoma cells. Concerning $CD8^+$ T cell, (E) gallbladder tumor was negative, while (F) lung tumor was scored as III (positive) (immunohistochemical stain, x100).

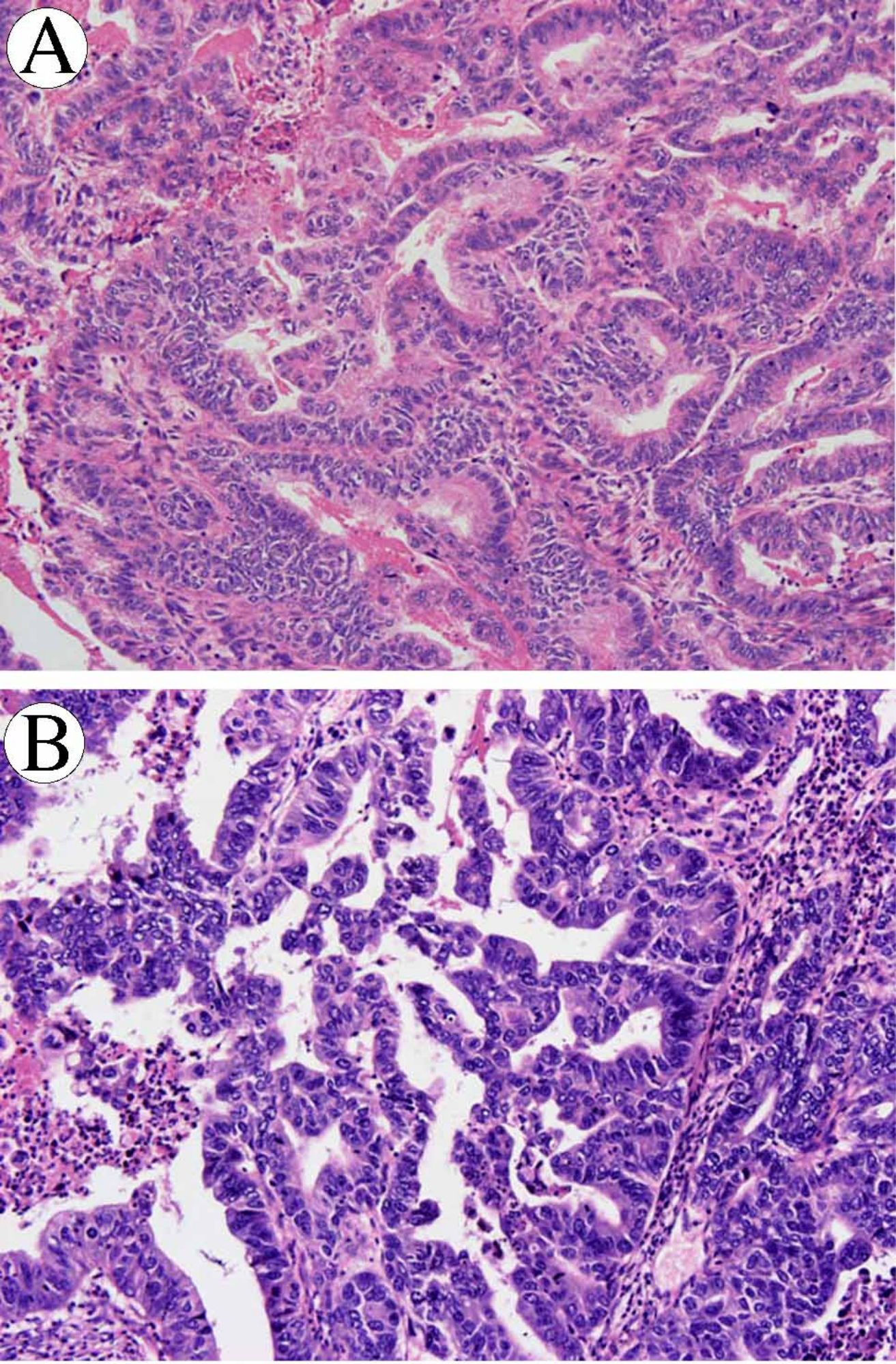

A
120kV/ 180m
0.75s/7m
-7.00m
R
B

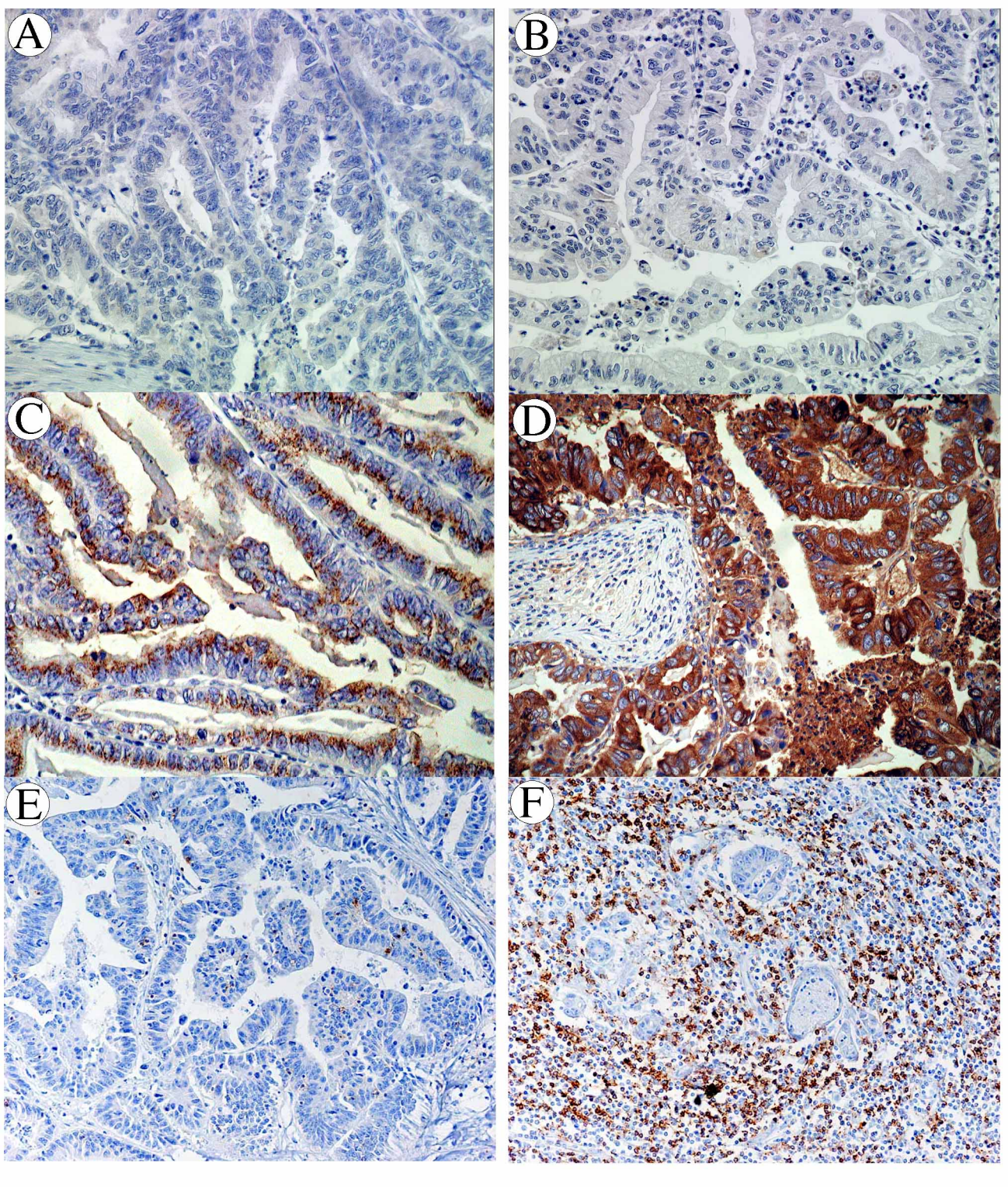

A
0.75s/7mm
-7.00mm/r
R
WL= -600
WW= 1600
63Y/F


B
2002.04.08 09:17:38.450
120kV/ 150mAs
7.00mm/r
HP1.0
R
WL=-650
WW=1600


A


B


A
B


A
B
C
D
E
F