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Mesothelin Expression Is a Prognostic Factor in Cholangiocellular Carcinoma

Ryohei Nomura[1], Hiroaki Fujii[2], Masaaki Abe[2], Hiroyuki Sugo[1], Yoichi Ishizaki[1], Seiji Kawasaki[1], Okio Hino[2]

[1]*Department of Hepatobiliary-Pancreatic Surgery and*

[2]*Department of Pathology & Oncology, Juntendo University School of Medicine, Tokyo, Japan*

Although mesothelin is highly expressed in epithelial mesotheliomas, and also in adenocarcinomas of the ovary and pancreas, the clinical significance of mesothelin in cholangiocellular carcinoma (CC) has not been reported, and its biologic features are largely unknown. In the present study, mesothelin expression was evaluated in 25 patients with CC using a well-characterized mesothelin monoclonal antibody (5B2). A total of 8 of the 25 patients with CC (32%) showed mesothelin immunoreactivity. The 25 patients were divided into 2 groups according to the percentage of tumor cells that were positive for mesothelin expression: negative (n = 17) or focally positive (mesothelin expression evident in less than 50%, n = 4; total, n = 21 for both groups), and positive (mesothelin expression evident in 50% or more, n = 4). The survival periods in both groups were statistically analyzed. The negative/focally positive group showed significantly longer postoperative survival than the positive group ($P = 0.006$). Also, mesothelin positivity was identified as an independent predictor of short postoperative survival. The present results suggest that mesothelin expression is a prognostic indicator in patients with CC.

Key words: Cholangiocellular carcinoma – Immunohistochemistry – Mesothelin – Survival period

The mesothelin gene encodes a 69-kd precursor that is proteolytically cleaved into an NH_2-terminal secreted form and a COOH-terminal membrane-bound form, 40-kd mesothelin, which is a glycosylphosphatidylinositol-linked glycoprotein.[1] Recently, mesothelin expression has been detected

Reprint requests: Ryohei Nomura, MD, PhD, Department of Hepatobiliary-Pancreatic Surgery, Juntendo University School Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan.
Tel.: +81 3 3813 3111; Fax: +81 3 3818 7589; E-mail: r-nomura@juntendo.ac.jp

in lung cancer,[2] uterine serous carcinoma,[3] and acute myeloid leukemia,[4] in addition to mesothelioma, ovarian cancer, and pancreatic cancer, which have been shown to have high mesothelin expression.[1,5–9] Little mesothelin expression has been detected in other types of cancers and normal tissues.[1,6,10] Argani *et al*[7] reported that all investigated pancreatic adenocarcinomas showed mesothelin expression, whereas normal pancreas tissue was negative.[7] In other previous studies, similar data have been confirmed by microarray, serial analysis of gene expression, U133 oligonucleotide array, and immunohistochemistry, suggesting that mesothelin expression may play an important role in pancreatic cancer tumorigenesis.[11,12] Ordonez[6] has estimated the immunohistochemical positivity of mesothelin expression in various kinds of tumors, including those of the hepatobiliary-pancreatic tract. However, among studies that have focused on mesothelin expression in the hepatobiliary-pancreatic tract, none have attempted to correlate mesothelin immunoreactivity with the clinicopathologic features of CC. Therefore, it has remained unclear how mesothelin acts during the process of tumorigenesis and whether it can be a useful marker for predicting the clinical outcome of CC. The aim of this study was to further investigate the frequency of mesothelin immunoreactivity and the intensity of its expression in CC, and to examine whether this marker has a utility for survival prognostication in patients with this carcinoma.

Patients and Methods

Tissue samples and patient characteristics

Between 1987 and 2003, 30 patients underwent hepatectomy for CC in our institute. No cases of hilar cholangiocarcinoma (Klatskin tumor) were included in the present series. A total of 3 absolutely noncurative patients and 2 patients whose prognosis was unclear were excluded from the study. The remaining 25 patients, in whom the tumors were completely resected by hepatectomy, were examined. There were 17 men and 8 women, ranging in age from 37 to 77 years (mean, 58.0 years). Final pathologic stages were determined according to The General Rules for the Clinical and Pathological Study of Primary Liver Cancer.[13] The final pathologic stages were categorized as follows: stage I (n = 2), stage II (n = 5), stage III (n = 3), stage IVa (n = 3), and stage IVb (n = 12). Informed consent was obtained from all patients. The research was undertaken in compliance with the Declaration of Helsinki.

Immunohistochemical techniques

Serial sections were cut at a thickness of 4 μm from representative formalin-fixed, paraffin-embedded tissue blocks. Sliced tissue sections were deparaffinized by gradual washes in xylene, then dehydrated in absolute ethanol. Any endogenous peroxidase activity was blocked by bathing in 3% H_2O_2 for 30 minutes, and then the slides were washed in running water. The mouse anti-human anti-mesothelin antibody 5B2 (Novocastra, Newcastle-upon-Tyne, United Kingdom) was used as a labeling marker for mesothelin. To retrieve the antigen epitope, we used a microwave processor with citrate-buffered solution, pH 6.0, at 95°C for 10 minutes. Protein blocking was achieved by incubation in 5% normal goat serum for 30 minutes. After microwave treatment, the sections were incubated with the 5B2 antibody (1:50) or negative control mouse serum at 4°C overnight. After rinsing in phosphate-buffered saline, the slides were treated with Envision+HRP-labeled (Dako Cytomation, Glostrup, Denmark) polymer anti-mouse immunoglobulin for 60 minutes. The peroxidase reaction was visualized by incubating the sections with 0.02%, 3,3′-diaminobenzidine tetrahydrochloride in 0.05 M Tris buffer, followed by counterstaining with hematoxylin.

Immunohistochemical evaluation

When the 5B2 signal was clearly demonstrated in the cytoplasm or membranes, cells were regarded as immunohistochemically positive. The results of mesothelin staining were classified as: negative, fewer than 1% of the tumor cells stained for mesothelin; focally positive, 1% to 50% of the tumor cells stained for mesothelin; and positive, more than 50% of the tumor cells stained for mesothelin.

Analysis of prognostic factors

Patients were followed for at least 5 years after surgery. Postoperative overall survival rates were compared between the mesothelin negative/focally positive group and the positive group. To evaluate several parameters affecting the length of postoperative survival, clinicopathologic features, such as vascular invasion and pathologic node, were also analyzed.

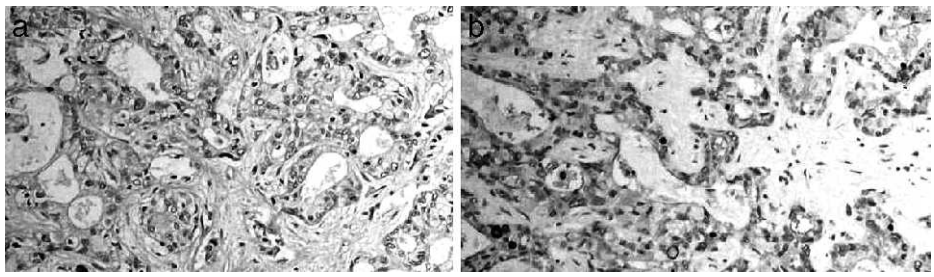

Fig. 1 Representative (a) hematoxylin and eosin staining (×400) and (b) mesothelin immunostaining (×400) in CC.

Statistical analysis

Survival curves were generated using the Kaplan-Meier method and compared using the log-rank test. In multivariate analysis, independent prognostic factors were determined by Cox proportional hazards model. Hazard ratio and 95% confidence interval were calculated as the measure of prognostic factors. Differences were considered statistically significant at $P < 0.05$.

Results

A total of 8 of the 25 patients with CC (32%) showed mesothelin immunoreactivity. In 4 of 8 patients, mesothelin expression was observed in less than 50% of the tumor cells (focally positive). In the other 4 patients, more than 50% of the tumor cells showed mesothelin expression (positive). The other 17 patients were negative. Thus, among the total patients examined, 21 were classified as negative or focally positive, and 4 were classified as positive. The negative or focally positive group was composed of 14 men and 7 women, with a mean age of 57.8 ± 13.5 years. The positive group was composed of 3 men and 1 woman, with a mean age of 59.5 ± 5.32 years. In relation to the cancer stage, stages I and II each included 1 focally positive patient. Stage III included 1 positive patient, and stage IV included 2 focally positive and 3 positive patients. Moreover, with the exception of the 17 negative patients, 5 of 8 (62.5%) patients presented with immunoreactivity for mesothelin belonging to stage IV. All of the positive patients showed mesothelin immunoreactivity in both the cytoplasm and membranes. Representative hematoxylin staining and mesothelin immunostaining in CC patients is shown in Fig. 1a and1b. Next, these CC patients were analyzed for correlation between mesothelin expression and patient survival. Figure 2 shows the postoperative survival curves of patients whose tumors were mesothelin negative or focally positive, and those whose tumors were positive, obtained using the Kaplan-Meier method. As can be seen, the group showing positive mesothelin expression (n = 4) had significantly shorter postoperative survival than the group showing mesothelin negativity or focal positivity (n = 21; $P = 0.006$). Results of univariate analysis of other prognostic factors for CC were as follows: patients with evidence of vascular invasion had significantly shorter survival than those without ($P = 0.028$; Fig. 3), and patients with pathologically evident lymph node metastasis also showed shorter survival than those without ($P = 0.012$; Fig. 4). Multivariate analysis using the Cox proportional hazards model identified mesothelin positivity as the sole predictor of short postoperative survival (Table 1).

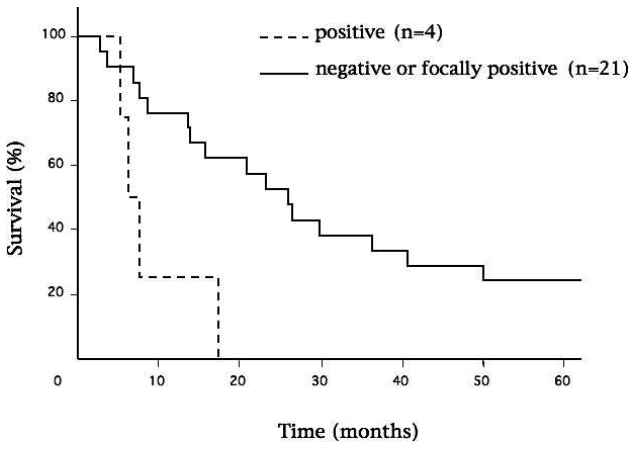

Fig. 2 The group with negative or focally positive mesothelin expression had significantly better survival than the positive group.

Discussion

Mesothelin has been a focus of attention since Chang *et al*[10] first reported its expression in 10 of 15 nonmucinous carcinomas of the ovary using the

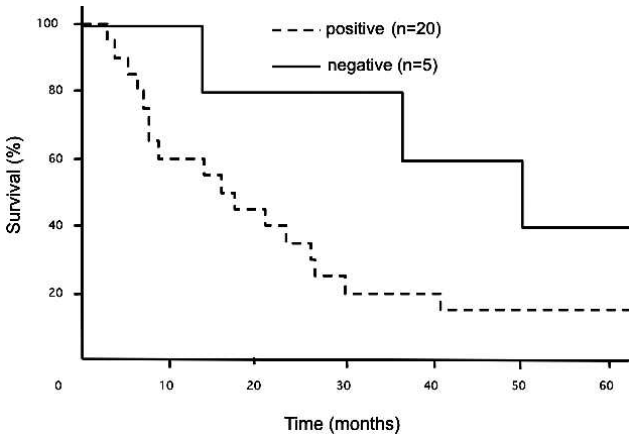

Fig. 3 Patients with evidence of vascular invasion had significantly poorer survival than those without.

K1 antibody in 1992. They also reported that mesothelin was expressed in mesothelial cells of the peritoneum, pleura, and other normal tissues, as well as in mesothelioma and squamous cell carcinoma.[1] Currently, it is generally agreed that the first choice of treatment for CC is surgical resection, although the results are not yet satisfactory. As for mesothelin expression in CC, only a few reports are available.[14] Therefore, it has been unclear how mesothelin is associated with the etiology of CC. Additionally, Ordonez *et al*[14] demonstrated that about one third of cholangiocarcinomas showed immunoreactivity for mesothelin. Thus, immunoreactivity for mesothelin in CC appears to be far lower than in pancreatic carcinoma, which affects the same hepatobiliary pancreatic structures. This result suggests that mesothelin plays a less important role in the differentiation process of CC than in that of

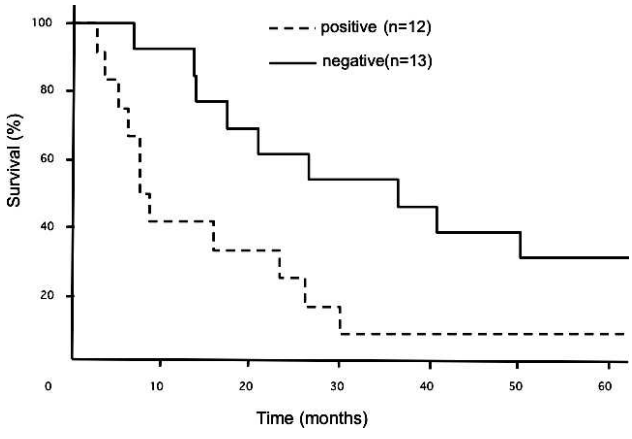

Fig. 4 Patients with pathologically confirmed lymph node metastasis also showed shorter survival than those without.

Table 1 Multivariate analysis using the Cox proportional hazards model identified mesothelin positivity as the sole predictor of short postoperative survival

	Hazard ratio	95% CI	P^a
Vascular invasion	1.44	0.344–6.002	0.620
Classification of pN	2.52	0.897–7.098	0.080
Mesothelin expression[b]	4.67	1.256–17.352	0.021

CI, confidence interval; pN, pathologic node.

[a]Cox proportional hazard test.

[b]For mesothelin expression, the patients were divided into 2 groups: a group with negative or focally positive expression for mesothelin, and a group with positive expression for mesothelin.

pancreatic carcinoma. In the present study, 32% of CC patients were immunoreactive for mesothelin. Nelson *et al* reported that 37% of cholangiocarcinomas showed mesothelin expression; hitherto, however, mesothelin expression has never been demonstrated in CC. Although in the present series of CCs the frequency of mesothelin expression was low, a proportion of patients did show some degree of mesothelin positivity, and data from this research indicated that higher mesothelin expression in the tumor cells was correlated with shorter patient survival. The function of mesothelin expression in CC tumorigenesis has been unclear, and some previous data conflict with those of the present study. Yen *et al*[15] showed that diffuse mesothelin expression was associated with a favorable clinical outcome in patients with high-grade ovarian serous carcinoma. The positive correlation between mesothelin expression and longer overall survival in patients with ovarian cancer is presumed to be due partly to a heightened immune response against mesothelin-expressing tumor cells.[15] Ho *et al*[2] reported a humoral response to mesothelin in patients with ovarian cancer and mesothelioma. They also found that immunogenicity of mesothelin was associated with high mesothelin expression in tumor cells. These findings indicate that an immune response against mesothelin-expressing ovarian carcinoma cells may result in a reduction of tumor load and contribute to prolongation of overall patient survival.[2,15] It has been reported that of the patients vaccinated with granulocyte macrophage colony-stimulating factor–transduced autologous pancreatic cells who developed systemic antitumor immunity, all had a strong mesothelin-specific $CD8^+$ T-cell immune response.[16] Furthermore, Hassan *et al*[9] have revealed a direct correlation between the generation of vaccine-dependent T-cell responses to mesothelin and long-term survival, making this a

promising approach for mesothelin-specific immunotherapy. They have suggested that a tumor containing a greater number of mesothelin-positive cells is more likely to elicit a response from the immune system, whereas a tumor with only focal mesothelin expression will tend to progress unchecked, because most tumor cells fail to express the tumor antigen. These findings suggest that mesothelin could have certain beneficial applications for cancer therapy. However, there are also some reports that support our present results. Li *et al* found that forced mesothelin expression significantly increased tumor cell proliferation and migration by 90% and 300%, respectively, and increased tumor volume fourfold in a nude mouse xenograft model in comparison with a vector control cell line. Silencing of mesothelin inhibited cell proliferation and migration in pancreatic cancer cells and arrested tumor progression in vivo, and the increases in mesothelin-specific antibodies and T-cell responses and the decrease in regulatory T cells were correlated with tumor progression and prolonged survival.[17] These findings emphasized the importance of mesothelin as a marker of biologic malignancy in pancreatic cancer from both genetic and immunologic viewpoints.[17] In the present study, we examined the immunohistochemical expression of CC, and our results indicated that the frequency of mesothelin positivity was almost equal to that in previous reports.[14] However, tumors with a lower percentage of mesothelin expression were associated with a significantly longer postoperative survival period than tumors showing higher expression. It is still unclear whether this is the reason for the malignant character of CC, or whether it is a result of malignancy. However, there might be some relationship between mesothelin expression and the process by which malignant characteristics are acquired. Further studies of the cellular localization of mesothelin in various tumors are needed. Normal mesothelial cells and mesothelioma cells show expression of mesothelin on their cell membrane.[6] Also, many pancreatic cancers show accentuated membrane expression of mesothelin.[7,8,11,14] Differences in the subcellular localization of mesothelin expression may be related to differences in the process of intracellular cleavage and glycophosphatidylinositol anchoring.[14,18] Overall, the available data suggest that mesothelin does exert some effect on the tumorigenesis of CC and could be applicable as a prognostic indicator. Furthermore, treatment that suppresses mesothelin expression, either immunologically or genetically, in mesothelin-expressing malignant tumors might in the future become an important therapeutic alternative for cases in which surgical treatment is insufficient.

Conclusion

In conclusion, mesothelin expression is a prognostic indicator in patients with CC.

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