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ORIGINAL PAPER

Immunohistochemical investigation of migration inhibitory factor-related protein (MRP)-14 expression in hepatocellular carcinoma

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Migration inhibitory factor-related protein (MRP)-8 and -14 belong to the S-100 protein family and are associated with myeloid cell differentiation. MRP is also expressed in some epithelia. However, there are few reports for the investigation on carcinomas.

Using the monoclonal antibody 60B8 against MRP-14, we carried out the immunohistochemical evalution of MRP-14 expression in 70 cases of hepatocellular carcinoma (HCC), and examined the relation to tumor differentiation and vascular invasion. Positively stained tumor cells were detected in 32 cases, all of which belonged to grade II (7/30) or grade III (25/25) of the Edmondson–Steiner classification. In particular, the grade III HCC showed a significantly greater positive reaction. Immunopositivity in the non-carcinomatous hepatocytes and bile duct epithelia was not observed.

These findings suggested that malignant hepatocytes newly express MRP-14 and that the neo-expression in differentiated HCC is related to the tumor differentiation and shows higher correlation in the poorly differentiated carcinomas. Furthermore, the cholangiocellular carcinoma and metastatic adenocarcinoma as control materials also presented a more marked immunoreactivity for MRP-14 in the poorly differentiated carcinomas, in a similar manner with the findings of the HCC. Accordingly, MRP is considered to be frequently neo-expressed in poorly differentiated carcinomas.

MRP-14 expression rate in the 48 HCC cases with vascular invasion was 56%, showing no significant difference compared with non-invasive tumors. *Medical Oncology* (2000) **17,** 183–188.

Keywords: MRP-14; hepatocellular carcinoma; S-100 protein; tumor differentiation; vascular invasion; immunohistochemistry; S-100A9

Introduction

Migration inhibitory factor-related protein (MRP) is the calcium-binding protein which belongs to an S-100 protein family.[1,2] This protein is also called S-100 A8·9, cystic fibrosis antigen, L1 antigen or calgranulin.[2–5] There are two kinds of MRP: one has a molecular mass of 8kDa

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(MRP-8), and the other has a mass of 14 kDa (MRP-14).[6] Both proteins are expressed in the lineage of myeloid cells.[2,3,6–11] Several studies on MRP suggested that this protein is regarded as a regulator of inflammation and is associated with myeloid cell differentiation.[6–12] MRP-8 and MRP-14 bind to cell membrane and cytoskeleton in a monomer form or in a form of non-covalently linked protein complexes in a Ca^{2+}-dependent manner.[2,9,10] Furthermore, recent studies have shown that MRP in myeloid cells is Ca^{2+}-dependently translocated from the cytoplasm to cell membrane and vimentin filament and is involved in the cytoskeletal-membrane interactions.[13] However, the biological functions of this protein are not clearly known.

The expression of this protein is also demonstrated in certain types of epithelia.[4,5,14] Goebeler *et al* indicated that MRP in epithelial cells is Ca^{2+}-dependently translocated to keratin filament and suggested that this protein is involved in the assembly or disassembly of intermediate filaments similar to the other members of the S-100 protein family.[14] In the previous reports, MRP expression in epithelial cells is limited to the squamous epithelia and squamous cell carcinoma.[4,5,14] The MRP expression has not been demonstrated in the epithelia of the non-squamous type in the normal condition.[4,5] Therefore, there are few reports for the investigation of MRP expression in specific carcinomas. Recently, the transcript of MRP-14 gene has been detected in cultured adenocarcinoma cells derived from human various organs.[15] This result suggests that MRP-14 is also neo-expressed in the epithelia of the non-squamous type in the cancerous condition.

In this study, we carried out the immunohistochemical evaluation of MRP-14 expression in hepatocellular carcinoma (HCC), and examined the relation to tumor differentiation and vascular invasion.

Materials and methods

Materials

1. Tissue specimens We retrieved 70 cases of primary HCC from surgical files at our hospital. All of these tumors were fixed in 10% formalin. From each of the 70 cases, an average of five blocks (ranging from 2 to 12, depending on the tumor diameter) were prepared, and the paraffin-embedded tissue sections were routinely stained with hematoxylin-eosin and reticulin silver impregnation.

Histological differentiation of HCC was graded following the Edmondson–Steiner (ES) classification.[16] Many HCC cases showed various degrees of differentiation in the same tumor. Such cases were classified into a grade which dominated in quantity, and only regions showing the graded differentiation were examined. According to the ES classification, the 70 HCC cases were classified into grade I ($n = 10$), II ($n = 30$), III ($n = 25$) and IV ($n = 5$). The liver tissues used as controls from the surgical files are enumerated as follows: cholangiocellular carcinoma (CCC $n = 12$, well differentiated; 3, moderately differetiated; 6, poorly differentiated; 3), metastatic adenocarcinoma from the stomach and large intestine ($n = 10$, well differentiated; 5, moderately differentiated; 5), hepatocellular adenoma ($n = 1$), focal nodular hyperplasia ($n = 2$), adenomatous hyperplasia ($n = 5$), liver cirrhosis ($n = 25$, viral; 23, alcoholic; 2) and non-cirrhotic liver tissues with or without chronic active hepatitis ($n = 20$). The latter three kinds of cases are derived from the non-cancerous parts in those HCC cases.

2. Antibody Establishment and characterization of the monoclonal antibody (mAb60B8) have been described elsewhere.[7,11]

Methods

1. Immunohistochemistry From each of the HCC cases, the representative tumor tissue sections, including the tumor centre and the invasion front, were examined. Deparaffinized tissue sections were trypsinized by incubation at 37°C for 20 min in 0.2% trypsin. Endogenous peroxidase was blocked by incubation for 15 min in the presence of methanol containing 0.3% hydrogen peroxide, followed by incubation for 10 min in the presence of 10% normal goat serum. Then these sections were incubated for 1 h with mAb60B8 (diluted at 1 : 1000) as the primary antibody. Immunohistochemical analysis was carried out by the streptoavidin–biotin technique using a kit HISTOFINE SAB-PO (MULTI) (Nichirei Co., Tokyo, Japan). The sections were then finally reacted in a 3, 3′-diaminobenzidin tetrahydrochloride substrate solution, counterstained with hematoxylin and mounted.

The tissue sections of the control materials were also immunostained in the same way.

2. Evaluation of MRP-14 expression MRP-14 expression was evaluated visually in two ways.[17] First, the percentage of positively stained tumor cells was estimated. When the immunopositivity was observed in the tumor cells, the feature was classified by the percentage of positive cells; less than 10% positive cells was designated as Score 1, greater than 10% but less than 50% as Score 2 and greater than 50% as Score 3. Secondly, the staining intensity in the tumor was estimated. The intensity of positivity was classified into the following three degrees. The tumor in which all positive cells showed weakly positive was designated as $+1$, the tumor in which tumor cells showed the staining intensity equivalent to that of infiltrating macrophages was designated as $+3$, and the intermediate staining intensity between the above two groups was designated as $+2$.

The control materials were also evaluated in the same way.

For statistical analysis, the χ^2 test was used, and $P < 0.05$ was considered significant.

Results

MRP-14 expression was immunohistochemically detected in the tumor cells in 32 of the 70 HCC cases. Inflammatory cells such as infiltrating macrophages, monocytes and neutrophils, showed strong positivity.

MRP-14 immunopositivity in each grade of tumor differentiation was 0/10 in grade I, 7/30 in grade II, 25/25 in grade III and 0/5 in grade IV, showing that all of the cases in which positively stained tumor cells were confirmed belonged to grade II or grade III (Table 1). Especially in grade III HCC cases, the positive tumor cells were observed in all cases, and there was a significant difference in the positivity rate between grade III HCC and grade II HCC ($P < 0.01$). Furthermore, the cases in which the percentage of positive tumor cells was scored 2 or 3, ie the percentage of positive tumor cells was greater than 10%, were limited to grade III HCC.

Table 1 Relationship between MRP-14 Immunoreactivity and the grades of histological differentation in HCC

	Histological grades			
MRP 14 immunoreactivity	*grade I (n = 10)*	*II (n = 30)*	*III (n = 25)*	*IV (n = 5)*
Non-reactive ($n = 38$)	10	23	—	5
Reactive ($n = 32$)	—	7	25	—
score 1	—	7	12	—
score 2	—	—	8	—
score 3	—	—	5	—

n: number of cases.

Immunoreactivity for MRP-14 was heterogeneous among the tumor cells. The staining intensity varied from absent to intense on the individual malignant hepatocytes in the tumor. Regarding all of the 32 cases in which postive tumor cells were confirmed, MRP-14 postivity in the tumor cells was observed in cytoplasm and nuclei, similar to that observed in inflammatory cells (Figures 1 and 2a). However, the positive staining was partially restricted to the cell membrance in some cells (Figure 2b). The distribution of these positive cells in the tumor tissues did not show any common tendency. The intensity of positivity was classified based on the differentiation grades in those 32 cases (Table 2). As shown in Table 2, a significant number of cases which were classified as an intensity of $+2$ or greater, belonged to grade III HCC ($P < 0.01$). Considering the relationship between the percentage of positive tumor cells and the intensity of positivity, the cases were divided into those which

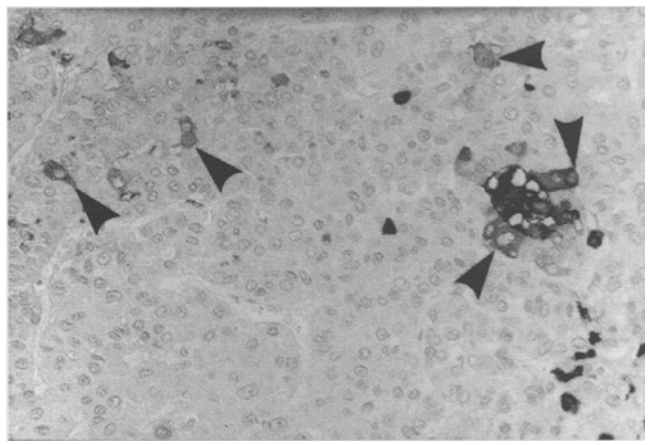

Figure 1. *Immunohistochemistry of MRP-14 in grade II HCC (×165). Positive staining was observed in a small number of the tumor cells forming trabecular or pseudoglandular structures (arrow heads). Infiltrating macrophotages showed strongly positive staining.*

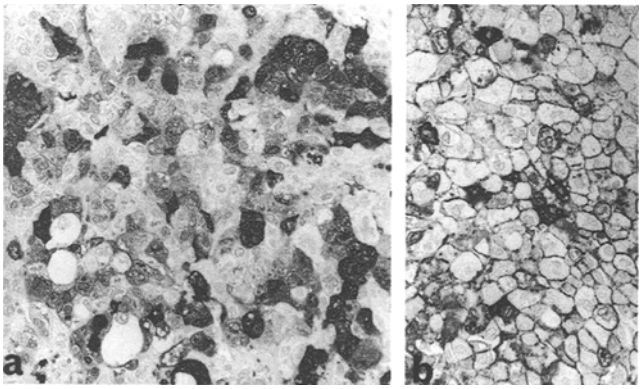

Figure 2. *Immunohistochemistry of MRP-14 in grade III HCC (×120). a: MRP-14 immunoreactivity was heterogeneous among the tumor cells. Cells showing strongly positive staining, weakly positive staining or no staining in the cytoplasm and nuclei, were intermingled. b: Positively stained substance was observed on the cell membrane in some tumor cells.*

Table 2 Relationship between the grades of histological differentiation and the intensity of positivity in the HCC expressing MRP-14

	Histological grades[a]	
Intensity of positivity	*grade II (n = 7)*	*grade III (n = 25)*
+1	5	8
+2	2	9
+3	—	8

[a] Grades I and IV were non-reactive.

Table 3 Relationship between the percentage of positive tumor cells and the intensity of positivity in the HCC expressing MRP-14

	Intensity of positivity	
Percentage of positive cells	*+1 (n = 13)*	*+2 and +3 (n = 19)*
Score 1 (*n* = 19)	11	8
Score 2 and 3 (*n* = 13)	2	11

scored 1 and those which scored 2 or greater and divided into those with an intensity of +1 and those with an intensity of +2 or greater. As a result, the cases scored 1 correlated with the cases showing an intensity of +1 while the cases scored 2 or greater related to the cases showing an intensity of +2 or greater ($P < 0.05$, Table 3).

In the cases used as controls, the immunopositivity for MRP-14 was detected only in the tumor cells of the CCC and metastatic adenocarcinoma, but not in the hepatocytes and bile duct epithelia in the non-cancerous condition, regardless of the presence or absence of the liver diseases. The MRP-14 positivity was 9/12 in the CCC and 9/10 in the metastatic adenocarcinoma. In the CCC, the number of cases which were scored more than 2 or showed an intensity of more than +2, were 0/3 in the well differentiated carcinomas, 1/6 in the moderately differentiated carcinomas and 3/3 in the poorly differentiated carcinomas. For the metastatic adenocarcinoma, the number of similar cases were 0/5 in the well differentiated carcinomas and 3/5 in the moderately differentiated carcinomas.

Vascular invasion was histopathologically observed in 48 of the 70 HCC cases, and the number of these cases in each grade of the tumor differentiation was 2/10 in grade I, 21/30 in grade II, 20/25 in grade III and 5/5 in grade IV. All seven cases of grade II HCC in which MRP-14 expression was detected were accompanied with vascular invasion. As shown in Table 4, the MRP expression rate in the 48 cases accompanied with vascular invasion was 27/48 (56%), showing no significant difference in MRP-14 positivity among the HCC cases accompanied with vascular invasion. Moreover, all of the CCC cases were accompanied by the vascular invasion regardless of the MRP-14 immunoreactivity.

Table 4 Relationship between MRP-14 immunoreactivity and vascular invasion in HCC

	Vascular invasion	
MRP-14 immunoreactivity	*Negative (n = 22)*	*Positive (n = 48)*
Non-reactive (*n* = 38)	17[a]	21[b]
Reactive (*n* = 32)	5[c]	27[d]
score 1	5	14
score 2	—	8
score 3	—	5

[a] Eight cases were grade I and 9 cases were grade II.
[b] Two cases were grade I, 14 cases were grade II and 5 cases were grade IV.
[c] Five cases were grade III.
[d] Seven cases were grade II and 20 cases were grade III.

Discussion

MRP antigen is resistant to formaldehyde and its presence can be demonstrated in routine pathological materials by immunohistochemistry.[4,11] The percentage of positively stained cells and the intensity of positivity were correlated in the HCC cases.

There are some reports on MRP expression in the epithelial cells in various organs, but MRP expression is not confirmed in the hepatocytes in the normal condition.[4,5] We also failed to find the immunopositivity in the non-cancerous hepatocytes regardless of the presence or absence of liver diseases. All HCCs in which positive tumor cells were shown belonged to grade II or III of the ES classification, and there were no positive tumor cells observed in grade I and IV HCC cases. These observations suggested that MRP-14 is not expressed in non-cancerous hepatocytes, well differentiated HCC cells or undifferentiated carcinoma cells. In contrast, in grade III HCC, the positive tumor cells were detected in the all cases. Furthermore, the positivity rate, ie the percentage of positive cells and the intensity of positivity, was significantly higher than that of grade II HCC. These results suggested that MRP-14 is neo-expressed in the malignant hepatocytes in grade II and grade III HCCs and that this expression in differentiated HCC correlates with tumor differentiation and shows higher correlation in the poorly differentiated carcinomas.

MRP expression is constantly observed in the squamous epithelia in such various conditions as normal, inflammation and carcinoma.[4,5] MRP expression in the glandular epithelial cells is not confirmed in the normal condition.[4,5] However, Matsumoto *et al* recently reported that transcript of MRP-14 gene is detected in the clutured human adenocarcinoma cells derived from various organs by reverse transcription-polymerase chain reaction.[15] In our study, the immunopositivity for MRP-14 was observed in the CCC cells and metastatic adenocarcinoma cells. Furthermore, the MRP-14 positivity in these tumor cells was also more conspicuous in the poorly differentiated carcinomas, in a similar manner with the findings of the HCC. As mentioned above, it is suggested that MRP is newly expressed in certain species of malignant epithelial cells and this neo-expression is more frequently shown in poorly differentiated carcinoma cells.

In the 48 HCC cases with vascular invasion, a significant difference in the MRP-14 immunopositivity was not detected. All of the CCC cases were accompanied by vascular invasion regardless of the MRP-14 immunoreactivity. Therefore, the MRP-14 positivity is considered to show little correlation with the vascular invasion of the carcinomas.

The function of MRP has not been clearly elucidated. This protein belongs to the S-100 protein family.[1,2] S-100 protein is known to be present not only in the cytoplasm but also in the nuclei in many tissues.[2] Furthermore, S-100 protein is considered to influence diverse cellular processes including cell cycle progression, cell differentiation, regulation of protein phosphorylation and cytoskeleton–cell membrane interactions.[1,2] It has been shown recently that some members of the S-100 protein family are up-regulated or down-regulated in tumor cells, which are related to cell differentiation, malignant transformation and cell cycle in tumor cells.[2] MRP is also considered to be involved in cytoskeleton–cell membrane interactions, and its intracellular localization is known to change depending on the intracellular Ca^{2+} conditions, which is a characteristic of the S-100 protein.[9,10,13] MRP is located predominantly in cytosol under low Ca^{2+} concentration and is translocated to cell membrane and cytoskeletal components under the condition of high Ca^{2+} concentration.[10,13] Immunohistochemical localization of MRP-14 on those HCC cells may represent the characteristic of this protein as a member of the S-100 family.

Using cultured squamous cell carcinoma cells, Goebeler *et al* reported that MRP in epithelial cells is translocated from the cytoplasm to cell membrane and keratin filament in a Ca^{2+}-dependent manner.[14] Furthermore, they indicated that the S-100 protein family is involved in the Ca^{2+}-dependent regulation of the intermediate filaments assembly.[14] It has been known that MRP in myeloid cells has a relationship with vimentin filament.[13] Although vimentin is considered an intermediate filament presented in mesenchymal cells,[18] this filament is also detected in poorly differentiated carcinoma and undifferentiated carcinoma.[19,20] In this study, we failed to find positive reaction in the tumor cells of grade IV HCC, that is, undifferentiated carcinoma cells, while MRP-14 expression was significantly detected in grade III HCC, that is, poorly differentiated carcinoma cells. It is difficult to interpret these results. Based on the

hypothesis[14] that MRP in epithelial cells is deeply involved in the assembly or disassembly of the keratin and vimentin filaments, it is supposed that MRP in carcinoma cells is newly expressed under a particular condition of the composition of these intermediate filaments. The composition of the intermediate filaments in carcinoma cells varies depending on the tumor differentiation and cell differentiation.[19,20]

The immunohistochemical results in our study can be understood as indicating that MRP expression in epithelial cells differs under the normal and cancerous conditions and that this expression varies based on the differentiation of carcinoma. Unlike squamous epithelial cells, MRP may be newly expressed in certain epithelial cells in relation to the changes in the intermediate filaments composition caused by cancerization and subsequent dedifferentiation. Furthermore, it was suggested that the MRP neo-expression is closely associated with biological malignancy of differentiated carcinoma such as tumor differentiation and cellular atypism.

Although HCC was used as the test materials in this study, we recently obtained similar results in lung adenocarcinoma (unpublished data). We believe the MRP expression in the other carcinomas is also valuable to investigate.

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