らに，SLC あるいは Mig を OVA と同時に発現する ES-DC は，OVA 単独発現の ES-DC よりも，抗腫瘍効果の誘導においても優れていた（図 6）．特に SLC の共発現により，最も強い抗腫瘍免疫の増強効果が得られた．

IX．アロマウス ES-DC を利用した腫瘍免疫の誘導および ES-DC の自己免疫疾患予防への応用

ES-DC を臨床応用しようとする場合，ES 細胞ドナーとレシピエントの間の HLA を初めとする遺伝的多型の差異に起因する組織不適合性の問題を解決する必要がある．しかしながら，マウスを用いた実験では，ES 細胞ドナーとレシピエントが同系でなくとも，MHC のアリルを一部共有していれば，共有された MHC クラス I 分子に提示された抗原により，抗原特異的な T 細胞を活性化し，抗腫瘍免疫効果も得られることを観察している[13]．筆者らは，ES-DC 技術の応用として，抗腫瘍免疫療法だけでなく，免疫抑制分子と自己免疫疾患の標的となる自己抗原を同時に強制発現させた ES-DC による，自己免疫疾患の予防にも成功している[14,15]．

X．ヒト ES 細胞からの樹状細胞の作製

筆者らは，ES-DC の臨床応用をめざして，ヒトの ES 細胞から ES-DC を分化誘導する方法を開発した．ヒトの ES 細胞は，マウスの ES 細胞に比べて上皮性の細胞に分化しやすい傾向があるが，マウスの場合と同様に OP9 細胞をフィーダー細胞として用いることにより，血液細胞を含む中胚葉系細胞への分化を誘導することが可能であった[16]．筆者らのヒトの ES-DC 分化誘導法では，培養系へ GM-CSF，M-CSF，および IL-4 を添加することにより効率良い分化誘導が可能であった．ヒト ES-DC もマウス ES-DC と同様に，タンパク質抗原をプロセスして T 細胞へ提示する活性や，アロ MLR 刺激活性など，樹状細胞としての機能を備えていた．また，マウス ES-DC の場合と同様の手法で，ヒトの遺伝子改変 ES-DC を作製することも可能であった．

おわりに

GPC3 由来の CTL エピトープは，HCC の免疫療法のあらたなターゲットとして，その臨床試験の結果が期待される．癌の免疫逃避に対抗するためには，多様な癌拒絶抗原のレパートリーを確立することが望まれる．GPC3 がその 1 つとして，HCC の再発および発症防止に寄与することを期待したい．

最近，ES 細胞で発現している数種類の遺伝子をマウスあるいはヒトの線維芽細胞等の体細胞に導入することにより，ES 細胞と同等の多分化能を有する iPS（induced Pluripotent Stem）細胞を作製できることが報告された[17,18]．iPS 細胞の作製には，ES 細胞の場合とは異なり，ヒト胚の滅失を必要としないという利点がある．さらに，iPS 細胞は，皮膚線維芽細胞など，比較的侵襲性の低い方法で採取できる細胞からも作製できるため，治療の対象となる患者自身など任意のドナーから作製することが可能である．ES 細胞から樹状細胞を作製する技術を iPS 細胞へ適用できれば，倫理的な問題を回避し，かつ，患者本人への負担を大幅に軽減しつつ治療に必要な樹状細胞を作製することが可能となる．著者らは，現在 iPS 細胞から樹状細胞を作製する研究も開始している．

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Glypican-3 is a useful diagnostic marker for a component of hepatocellular carcinoma in human liver cancer

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Abstract. Primary liver cancers are classified into three types based on their morphology and cytogenetic characteristics hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC) and combined hepatocellular and cholangiocarcinoma (CHC). It is often difficult to distinguish these liver tumors. Glypican-3 (GPC3) is serological and histochemical marker of hepatocellular carcinoma. In order to separate these three types of liver cancers, we analyzed the GPC3 expression in 85 liver resection specimens, including 46 HCCs, 28 ICCs and 11 CHCs. GPC3 immunohistochemical staining was used to distinguish HCC from ICC by comparing with the conventional biomarker, α-fetoprotein (AFP). The immunostaining of GPC3 was identified in 78.3% (36/46) of HCCs, 60% (9/15) of well differentiated, 88.9% (16/18) of moderately differentiated and 84.6% (11/13) of poorly differentiated HCCs. It was negative in the ICCs. We confirmed that GPC3 expression is specific to HCC component (8/11, 72.7%) but few samples also showed weakly in ICC component (2/11, 18.2%) of CHC sections among 11 cases compared with HCC biomarkers including AFP and hepatocyto paraffin 1 (HepPar1), and ICC biomarkers cytokeratin (CK) 7 and CK19. Three cases in which the macroscopic features resembled ICC did not express GPC3 even in the pathological HCC component. Most (10/11, 91%) of the pathological cholangiocarcinoma components in CHC showed positive staining for CK7 and CK19. The results of this study suggest that GPC3 is a biomarker that is sensitive and specific to HCC component of CHC, and CK7 and CK19 are markers for pathological cholangiocarcinoma component of CHC.

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Abbreviations: HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; CHC, combined hepatocellular and chorangiocarcinoma; GPC3, glypican-3; AFP, α-fetoprotein; HepPar1, hepatocyto paraffin 1; CK, cytokeratin; CC, cholangiocarcinoma; cp, component

Key words: hepatocellular carcinoma, intrahepatic cholangiocarcinoma, combined hepatocellular and chorangiocarcinoma, glypican-3, CK7, CK19, immunohistochemical analysis

Introduction

Liver cancer is one of the common malignancies that are rapidly increasing throughout the world. Primary liver cancers are classified into three types based on their morphology and cytogenetic characteristics, hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma (ICC) and combined hepatocellular and cholangiocarcinoma (CHC). HCC is hepatocyto-origin, and ICC is from the epithelium of the intrahepatic bile duct. CHC is a rare type of liver cancer with features of both hepatocellular and biliary differentiation (1-3). The pathological structure of CHC is composed of hepatocellular element showing bile production, an intercellular bile canaliculi or trabecular growth pattern and cholangiocellular component showing mucin production or gland formation.

Because of their rapid growth rate and the lack of accurate ways of diagnosis in the early stages, the prognosis and the survival rate for liver cancer patients remain poor. Currently, ultrasound sonography (US), computed tomography (CT), magnetic resonance imaging (MRI), and histopathological examination for tumor biopsy are used for diagnosis. However, distinguishing the three different primary liver tumors is often a challenging task in diagnosis, for which immunohistochemical analysis for specific antigens is a helpful tool: α-fetoprotein (AFP) and hepatocyto paraffin 1 (HepPar1) for HCC (4-8) and cytokeratin (CK) 7 and CK19 for ICC (9-11).

Glypican-3 (GPC3) was discovered as a potential serological and histochemical marker whose expression is specific for HCC (12-16). GPC3 belongs to glypican family that is a group of heparan sulfate proteoglycans linked to the outer surface of cell membrane through a glycosylphosphatidylinositol anchor (17). In mammals, six members of GPCs have been reported, GPC1 to GPC6. GPCs are released from the cell surface by a lipase called Notum to regulate the signaling of Wnts, Hedgehogs, fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) (18-25). Depending on the cellular context, their function can be stimulatory or inhibitory activity, or signaling. The expression of GPC3 is detected in placenta and fetal liver, but not in other normal organs. During hepatic carcinogenesis, GPC3 have been reported to reappear in HCC and to be released into serum (12,13,15,26). Its expression is also detected in melanoma (27-29). The functions of GPC3 in cancer cells are still unclear.

In this study, we examined whether immunohistochemical analysis for GPC3 can be used to distinguish HCC from ICC, if so, how effectively GPC3 can be detected, compared to other biomarkers that are conventionally used. We demonstrate that distinguishing HCC from ICC by detecting the expression of GPC3 enables more accurate diagnosis.

Materials and methods

Case selection. We selected 85 cases of liver tumors from the surgical pathology files from 1992 to 2006 of National Cancer Center Hospital East, Kashiwa, Chiba, Japan. The cases included 46 primary HCCs, 28 ICCs, and 11 CHCs that underwent hepatectomy. All identifiers were eliminated to protect patients' identities. Size of the tumor and any clinicopathologic factors (age, sex and grade of tumor) were matched between HCC and ICC. The 46 cases of HCCs occurred in 33 men and 13 women with a mean of age at 65.3 years (range, 44-80 years). HCC was subclassified into well (n=15), moderately (n=18), and poorly (n=13) differentiated types according to the World Health Organization classification criteria. The 28 cases of ICC consisted of 18 men and 10 women. Their mean age was 65.7 years (range, 51-82 years). All 28 resected cases of ICC were confirmed by hematoxylin-eosin (H.E.) staining.

The 11 cases of CHC included 7 men and 4 women with a mean age of 62.5 years (range, 47-76 years). All CHCs were pathologically confirmed after surgery.

Tissue samples. Liver tissue sections were retrieved from the files of the Department of Pathology in our institution. All liver specimens were prepared from surgically resected tumors and adjacent parenchyma. They were fixed in 10% formalin and paraffinized for routine histological examination.

Immunohistochemical staining procedure. Six-micrometer-thick sections were made from the paraffin-embedded blocks. Subsequently the sections were deparaffinized in xylene and rehydrated through ethanol to water. Endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol for 20 min. For antigen retrieval, Sections were heated in 10 mM citrate buffer (pH 6.0) with microwave for 15 min in a water bath at 95˚C. Only for CK7 immunostaining, sections were digested by Proteinase K (DakoCytomation, Carpenteria, CA) for 5 min at room temprature. Slides were then allowed to cool down. The prediluted primary antibodies, monoclonal anti-GPC3 (dilution 1:300, 1G12; Biomosaics, Inc., Burlington, VT), anti-AFP (dilution 1:400, DakoCytomation), anti-HepPar1 (dilution 1:100, DakoCytomation), anti-CK7 (dilution 1:100, DakoCytomation), and CK19 (dilution 1:200, DakoCytomation) were added to cover each slide, and the slides were incubated for 2 h at room temperature. Slides were washed 3 times in phosphate-buffered saline (PBS)/Tween for 5 min each. Mouse Envision Polymer (DakoCytomation) was used as a secondary antibody for 30 min at room temperature followed by washes in PBS/Tween 3 times for 5 min each. Diaminobenzidine chromagen (DakoCytomation) was added to each slide and incubated for 2 min. Slides were washed in distilled water, counterstained with hematoxylin and dehydrated in xylene. To analyze GPC3 expression, the immunohistochemical results were classified according to the number of positive cells as follows: -, negative (<10%); ±, weakly positive (10-30%); + positive (>30%). To validate the data in GPC3 as a marker for HCC, parallel staining for AFP of 46 cases were further analyzed. For 11 CHC cases, AFP, HepPar1, CK7 and CK19 were stained and compared with GPC3 staining pattern.

The slides were examined independently by 3 observers (Shirakawa H, Kuronuma T and Nakatsura T) and then collectively by 2 more pathologists (Hasebe T and Nakano M).

Statistical analysis. Differences in proportion were tested by the χ^2 test. Differences in the means of each subgroup were tested using the Student's t-test. P-value of <0.05 was considered statistically significant.

Results

GPC3 was present in 80% of HCC and negative in ICC. In order to examine the levels and pattern of GPC3 expression, 46 cases of HCC and 28 cases of ICC were immunohistochemically analyzed. GPC3 was detected in 36 cases (78%) of HCC (Fig. 1a), and no expression of GPC3 was found in any of the ICC patients (Fig. 1b). The GPC3 staining was diffused throughout (Fig. 1c) or localized in a granular pattern in the cytoplasm (Fig. 1d). In other cases, GPC3 was observed at the plasma membrane (Fig. 1e). Previously GPC3 is shown to bind to the cell membrane (16), however, those cases with membranous GPC3 had staining in the cytoplasm as well, but there was no case of GPC3 located only at the plasma membrane. When sensitivity of GPC3 was evaluated, 36 cases (78%) were positive for GPC3 when only 16 cases (35%; P<0.0001) were stained for AFP in HCC suggesting that GPC3 is more sensitive than AFP. Thus, GPC3 was confirmed to be specific and sensitive to HCC compared to AFP.

GPC3 expression increased in moderately and poorly differentiated HCC. In terms of GPC3 expression and tumor

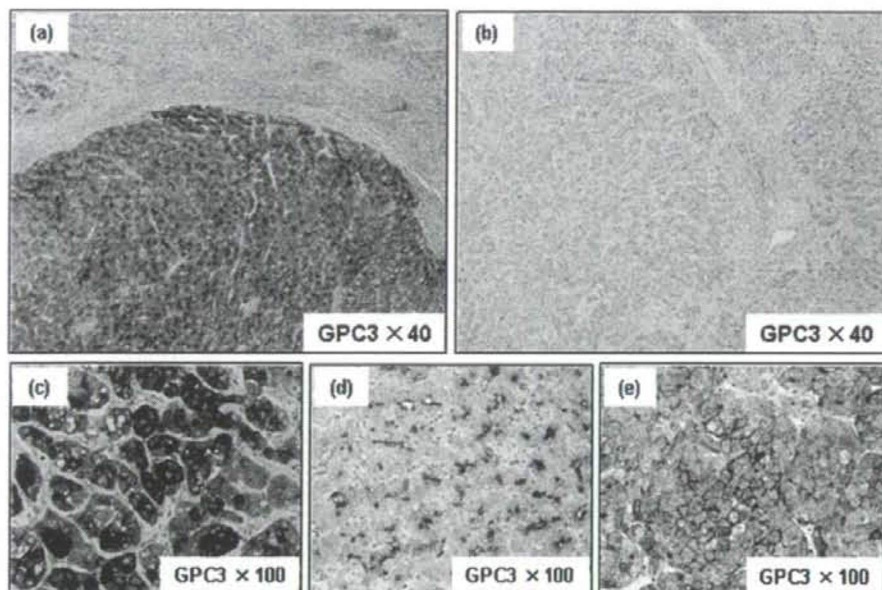


Figure 1. GPC3 expression was specific to HCC and absent in ICC. Immunohistochemical detection of GPC3 expression in HCC (a) and ICC (b) (magnification, x40). Immunostaining patterns of HCC: (c) diffuse in cytoplasm, gulanular in cytoplasm (d), and membranous (e).

Table I. Correlation of positive for GPC3 staining and tumor grade.

Grade of tumor	HCC					ICC		P-value
	No. of case	GPC3 -	GPC3 ±	GPC3 +	GPC3 positivity	No. of case	GPC3 positivity	
Well-differentiated	15	6	5	4	9 (60%)	8	0 (0%)	
Moderately differentiated	18	2	4	12	16 (89%)	10	0 (0%)	
Poorly differentiated	13	2	5	6	11 (85%)	10	0 (0%)	
Total	46				36 (78%)	28	0 (0%)	<0.0001

-, negative (<10%); ±, weakly positive (10-30%); +, positive (>30%).

differentiation level, GPC3 was expressed in 9 (60%) of 15 well differentiated, 16 (89%) of 18 moderately differentiated and in 11 (85%) of 13 poorly differentiated HCC (Table I). AFP was expressed in 3 (20%) of 15 well differentiated, 6 (33%) of 18 moderately differentiated and in 7 (54%) of 13 poorly differentiated HCC (data not shown). The expression level of GPC3 was lower in well differentiated HCC than in the other HCC grades, though the difference was not statistically significant (well- vs. moderately differentiated: P=0.054, well- vs. poorly differentiated: P=0.150). Thus, GPC3 expression is also a good indicator for malignancy levels.

GPC3 expression was observed specifically in pathological HCC component in CHC. There are discrepancies between preoperative diagnosis and pathological findings for CHC patients. Diagnostic results and the expression of tumor markers of 11 CHC patients are summarized in Table II. Initial diagnosis was carried out by H.E. staining. Among these 11 patients, 7 patients (63.6%) were diagnosed as HCC and 3 (27.3%) were ICC. Only 1 patient (9%) of the 11 CHC was correctly diagnosed as CHC. To seek the possibility to use GPC3 immunostaining to detect HCC component (cp) in CHC, combination of antibodies against GPC3, AFP, HepPar1, CK7 and CK17 were used. In addition to AFP, HepPar1 is frequently used as marker for HCC (4-8) and CK 7 and CK19 for ICC (9-11).

Among 11 CHC cases, 4 cases preoperatively diagnosed as HCC were chosen to represent the collision and transitional type of CHCs based on the macroscopic features

Table II. Correlation of immunostaining varieties and pathological components of CHC.

Pt. no.	Preoperative diagnosis	Macroscopic diagnosis	Pathological hepatocellular carcinoma component					Pathological cholangiocarcinoma component				
			GPC3	AFP	HepPar1	CK7	CK19	GPC3	AFP	HepPar1	CK7	CK19
1	HCC	CHC	+	+	-	+	+	-	-	-	-	-
2	HCC	HCC	+	-	-	-	-	-	-	+	+	+
3	HCC	HCC	+	-	+	-	-	±	-	-	+	+
4	CHC	HCC	+	+	+	-	-	±	-	-	+	+
5	HCC	CHC	+	-	+	-	-	-	-	-	+	+
6	HCC	CHC	+	-	-	-	-	-	-	+	+	+
7	ICC	CHC	±	-	-	±	+	-	-	-	+	+
8	HCC	HCC	+	+	-	-	-	-	+	-	+	+
Total ± positive rate (%)			8/8 100	3/8 38	3/8 38	3/8 38	2/8 25	2/8 25	1/8 13	2/8 25	7/8 88	7/8 88
9	ICC	ICC	-	-	-	-	-	-	-	-	+	+
10	HCC	ICC	-	-	-	+	±	-	-	-	+	+
11	ICC	ICC	-	-	-	+	+	-	-	-	+	+
Total ± positive rate (%)			0/3 0	0/3 0	0/3 0	2/3 67	2/3 67	0/3 0	0/3 0	0/3 0	3/3 100	3/3 100

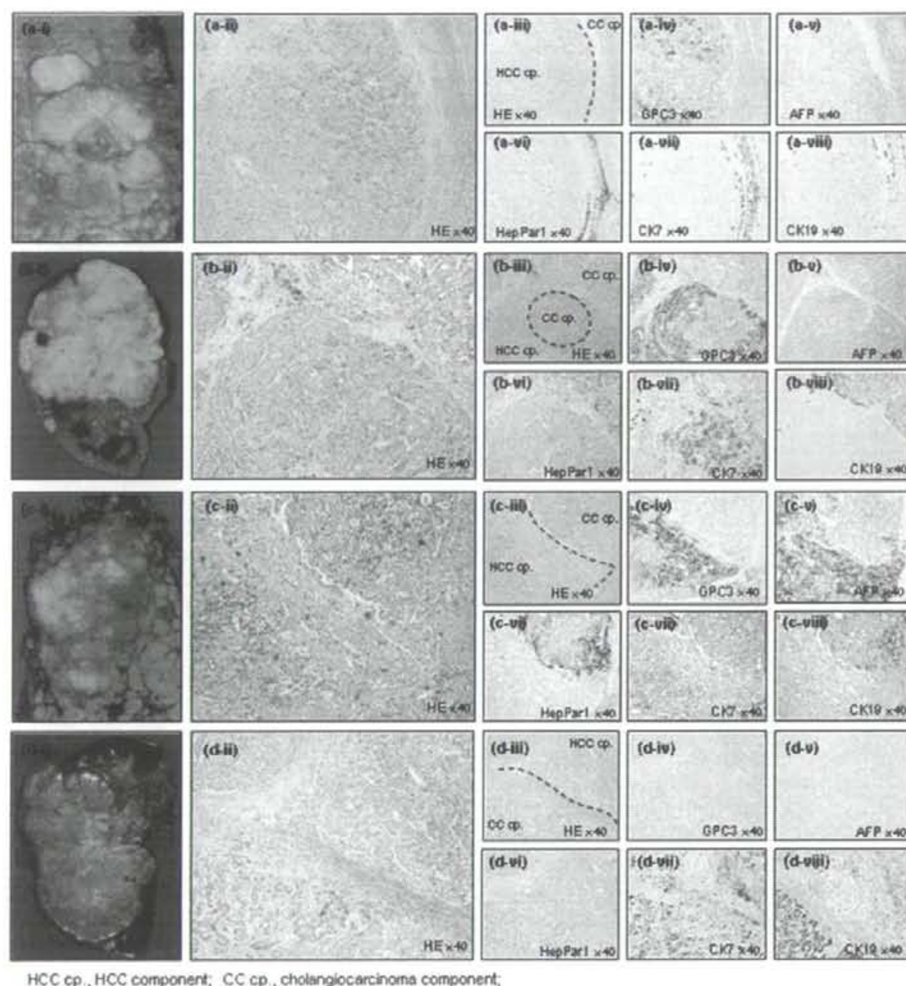
-, negative (<10%); ±, weakly positive (10-30%); +, positive (>30%); HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; CHC, combined hepatocellular and cholangiocarcinoma; GPC3, glypican-3; AFP, α-fetoprotein; HepPar1, hepatocyto-paraffin 1; CK, cytokeratin; CC, cholangiocarcinoma.

in cut surface. In Fig. 2, macroscopic observation and the immunostained histological sections are shown. These sections include 2 elements with pathological HCC cp forming bile production and trabecular growth pattern by eosinophilic staining and cholangiocarcinoma (CC) cp forming mucin production or gland formation by basophilic staining. Cases 1-8 were GPC3 positive, and cases 9-11 were negative for GPC3 in the HCC cp. Macroscopic, histological and immunohistochemical features of cases 2, 6, 8 and 10 are shown in Fig. 2a, b, c and d. Case 2 had greenish white and yellow nodules within the same tumor mass in the cut surface. HCC subtypes such as simple nodular and confluent multinodular type exist. Case 2 exhibited the features of HCC with multinodular type (Fig. 2a-i). Pathological diagnosis by H.E. staining revealed CHC pathologically (Fig. 2a-ii and -iii), which was so-called 'collision'-type tumor as reported by Goodman *et al* (30). A 'collision'-type tumor is coincidental occurrence of HCC and CC within the same tumor mass (31). GPC3 was positive (Fig. 2a-iv), but AFP and HepPar1 were not detected in HCC cp (Fig. 2a-v and -vi). Although HepPar1 is generally used as HCC marker, it was unexpectedly stained in CC region as well as CK7 and CK19 (Fig. 2a-vii and -viii).

Case 6 showed pale and lobulated phenotype in the cut surface macroscopically (Fig. 2b-i), and pathological diagnosis was also confirmed by H.E. staining (Fig. 2b-ii and -iii). This was so-called 'transitional' type tumor (30). A 'transitional' type tumor has an area of HCC that appears to transform into CC (31). GPC3 was stained in pathological HCC cp (Fig. 2b-iv) where AFP was negative (Fig. 2b-v). The HCC region was surrounded by pathological CC cp with the staining for CK7 (Fig. 2b-vii). HepPar1 and CK19 were detected in the same region with CC cp (Fig. 2b-vi and -viii). HepPar1 stained the CC cp as in case 2. The immunoreactivity of CK19 was not consistent with that of CK7.

Case 8 was diagnosed as HCC similarly to cases 2 and 6, but mixed tumor masses with white and gray in the cut surface were observed (Fig. 2c-i and c-ii). Both GPC3 and AFP were positive in HCC cp (Fig. 2c-iv and -v). HepPar1 was stained in CC cp (Fig. 2c-vi). CK7 and CK19 were positive in CC cp (Fig. 2c-vii and -viii), especially CK19 was more specific for CC cp than CK7. These three cases (cases 2, 6 and 8) indicated that detecting GPC3 can compensate for AFP and enhance the ability to identify the presence of HCC cp in CHC.

Cases 9, 10 and 11 were negative for GPC3 expression in several tumors. Macroscopically, they had the features of ICC with irregular shaped, white solid tumor masses. As an example, case 10 is shown in Fig. 2d. Althogh case 10 was diagnosed as HCC preoperativerly, it showed macroscopic freatures of ICC with the presence of abundant fibrous stroma and indistinct tumor margin (Fig. 2d-i). This case was later diagnosed as CHC based on the pathological examination (Fig. 2d-ii and d-iii). GPC3, AFP and HepPar1 were not detected in either HCC cp or CC cp (Fig. 2d-iv, -v, and -vi). CK7 was stained diffusely in the tumor (Fig. 2d-vii), and CK19 expression was more specific in CC cp than CK7 (Fig. 2d-viii). These 3 cases showed positive staining



HCC cp., HCC component; CC cp., cholangiocarcinoma component;

Figure 2. Macroscopic, histological and immunohistochemical features of four cases of CHC, a, case 2; b, case 6; c, case 8; d, case 10 in Table II. (a-i) Macroscopic feature in cut surface of case 2 tumor. (a-ii) The histological structure can be also devided into 2 types. HCC component showed expansive growth oppressing the cholangiocarcinoma component. (a-iii) Collision border between hepatocellular carcinoma and cholangiocarcinoma component are indicated as dots. The tumor cells within mainly hepatocellular carcinoma component showed only expression of GPC3 (a-iv) without expression of AFP (a-v). In the opposite side, the glandular area with cholangiocarcinoma component shows HepPar1 (a-vi), CK7 (a-vii) and CK19 expression (a-viii). (b-i) Case 6 shows macroscopic CHC feature in tumor cut surface that was suspected out HCC preoperatively. (b-ii) The histological cholangiocarcinoma component forming trabeculae with columnar appearance was surrounded by HCC component forming hepatoid structure. (b-iii) A dotted line is a boundary of HCC in the H.E. staining. The tumor cells within transitional region were positive for GPC3 (b-iv), CK 7 (b-vii) and CK 19 (b-viii). The difference was recognized between hepatocellular carcinoma component and cholangiocarcinoma component because GPC3 positive area encircled the CK7 area. The expressions of AFP (b-v) and HepPar1 (b-vi) were not observed. (c-i) Though case 8 was also suspected to be HCC preoperatively, the macroscopic features showed atypical HCC with mixed white and gray and indistinct tumor border. (c-ii) The cholangiocarcinoma component was obviously composed of structural gland formation. (c-iii) Collision area was distinguished histopathologically by a dotted line. The tumor cells of HCC component showed not only GPC3 (c-iv) but also AFP expression (c-v). In the glandular area of cholangiocarcinoma component, HepPar1 was expressed (c-vi), but CK7 not at all (c-vii) and CK19 shows weak positive expression (c-viii). (d-i) Case 10 shows macroscopic ICC features in tumor cut surface that was suspected as HCC preoperatively. (d-ii) The histological structure can be devided into 2 types with cholangiocarcinoma component forming trabeculae with columnar appearance and HCC component forming hepatocellular structures. (d-iii) A dotted line is a boundary of HCC in the H.E. GPC3 (d-iv), AFP (d-v) and HepPar1 (d-vi) were not stained, but CK7 (d-vii) and CK19 (d-viii) stained the cholagiocarcinoma component.

for CK7 and CK19 in CC cp, but not AFP or HepPar1 in HCC cp. Therefore, accuracy of CHC diagnosis can be achieved by combination of multiple tumor markers in addition to morphological characteristics: GPC3 that is specific for pathological HCC cp of CHC, and CK7 and CK19 that are specific for pathological CC cp of CHC.

Discussion

The diagnosis for HCC, ICC and CHC has been routinely performed by histopathological examination. Additionally, diagnosis of HCC is done by supplementary immunohistochemical analysis for AFP and HepPar1. Until now, though

the sensitivity is limited. AFP has been regarded as the most useful marker for HCC (4,32-34). HepPar1 is also widely used for HCC to distinguish between primary HCC and ICC. However, both markers are limited for the ability to discriminate different levels of malignancy in HCC because its sensitivity drops substantially in poorly differentiated HCC, and it does not discriminate between benign and malignant liver cancers (35). As these biomarkers frequently results in misdiagnosis, in this study, we showed that GPC3 is more sensitive to detect HCC compared to AFP. Due to the fact that GPC3 was downregulated in ICC (36), GPC3 may help to separate HCC from ICC.

CHC is the least common primary cancer of the liver but followed by an aggressive growth, it tends to metastasize to many organs leading to significantly poorer prognosis than HCC and ICC (31,37,38). Correct diagnosis leads to both appropriate treatment and better outcome for the patients. Nishie, *et al* reported that one third (nine of 27 cases) of patients with CHC were correctly diagnosed by enhanced computed tomography (39). In our study, only one of the 11 (9.1%) patients with CHC was correctly diagnosed before operation without fine needle aspiration biopsy. The difficulty to pathologically distinguish CHC from HCC and ICC comes from glandular or pseudoglandular structures in HCC and solid or trabecular patterns in CC (37,38). We believe that combination with histopathological examination with GPC3 immunostaining and radiological examination can bring an accurate diagnosis and improved clinical therapies for the patients leading to a better prognosis.

We showed that the immunostaining for GPC3 is specific for HCC patients and not detected in ICC patients. This confirmed that detecting GPC3 may improve the method to diagnose CHC. Of the 11 cases of CHC, 8 displayed GPC3 expression in restricted area of HCC cp. We demonstarated that immunohistochemical staining of GPC3 in liver tumor helps to recognize the pathological HCC cp more precisely. GPC3 expression was observed with high frequency in the HCC cp compared with AFP and HepPar1. HepPar1 was unexpectedly stained in CC cp, but this has been observed previously as well (7,40). This could be due to a transition from HCC to ICC where HepPar1 is one of the molecules that is downregulated at later stages in the process. CK7 and CK19 have been already reported as good markers of biliary epithelial differentiation (41). These were highly expressed in pathological CC cp (10/11, 91%) in CHC. The positive immunoreactivity of CK19 was more distinct than that of CK7 whose staining was weaker. Our immunohistochemical data disclosed that GPC3 can be a better marker specific for HCC leading to a better confirmation for HCC component of CHC as well as for HCC. Moreover, it provided evidence of the biologic behavior of such combined tumors, which are phenotypically and genetically leaning toward either ICC with predominant biliary differentiation or HCC with hepatocellular differentiation (42,43).

Employing multiple tumor markers may also allow the accurate diagnosis of CHC containing both hepatocellular and biliary differentiation. Concerning sensitivity and specificity, the combination of GPC3 for HCC cp and CK19 for ICC cp seems to be useful in the diagnosis of liver cancer. For CHC, GPC3 positive/CK19 negative profile suggests HCC, GPC3 positive/CK19 positive indicates CHC, and GPC3 negative/CK19 positive essentially rules out HCC and suggests the possibility of CC or CHC.

We developed a new anti-cancer immunotherapy with GPC3 as a target (44-47), and the phase I clinical trial of GPC3-derived peptide vaccination for advanced HCC is now on going. Because this new immunotherapy is not indicated for ICC, immunohistochemical staining of GPC3 is a useful method to select eligible patients. Furthermore, if CHC would be justified as a target of our immunotherapy in future, immunohistochemical analysis for GPC3 expression is indispensable for the process of patient selection.

GPC3 is expressed in the group of cells that are AFP-positive and/or CK7/19-positive in injured livers with activation of oval cell compartment; an indication for liver repair and regeneration (48). In addition, CK7, CK19 and AFP are frequently expressed in biliary epithelial cells (49,50) and in immature fetal hepatoblasts (51,52). Liver progenitor cells originate from the canal of Hering, lined by both hepatocytes and biliary ductular epithelial cells (53). It is not clear whether GPC3 is expressed in hepatic embryonic progenitor cells or cancer stem cells, but GPC3 may be a marker for hepatic progenitor/stem cells. In CHC cases of 2, 3 and 4, GPC3, CK7 and CK19 coincided in the regions of HCC and CC. Although HCC and ICC are two different kinds of primary liver malignancies arising from different cell types as hepatocytes and cholangiocytes, co-localization of GPC3 and CK7/19 suggest that the CHC is originated from progenitor or oval cell. In addition, case 6 showed an HCC lesion with GPC3 positive immunostaining surrounded by CC (Fig. 2b). This finding suggests that GPC3-positive HCC tumor cells are derived from GPC3-negative CC mass. Moreover, we predict from the fact that GPC3 is expressed in embryonic liver and downregulated after birth in normal liver but reappears in cancer is due to its regulatory role in proliferative and dedifferentiated cells, like cancer cells that acquired a progenitor- or cancer stem cell-like characteristics.

In summary, we confirmed that GPC3 is a marker sensitive and specific for HCC, but not ICC. Moreover, we revealed that GPC3 was expressed specifically in the HCC cp in the CHC. Therefore, GPC3 is a molecule that is significant not only in clinical but also biological field. It is clinically an important biomarker that can be used for accurate diagnosis leading to a better treatment and prognosis. Also, biologically, it may be an indicator for the identity and the origin of the cancer cells.

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Differential expression of heat shock protein 105 in melanoma and melanocytic naevi

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The objective of this study is to assess the expression of heat shock protein 105 (HSP105) in melanoma and benign melanocytic lesions. The expression of HSP105 in 62 human melanoma samples – 46 primary and 16 metastatic lesions – and 42 melanocytic naevi samples, was assessed by immunohistochemistry. Western blotting was performed on melanoma cell lines, melanoma tissues with matched normal skin and melanocytic naevi. The Mann–Whitney test was used for statistical analysis and significance was considered to be *P* less than 0.05. Seventy-four per cent of the primary melanoma lesions and 88% of the metastatic lesions overexpressed HSP105 by immunohistochemistry. The majority of melanocytic lesions (95%) were negative ($P<0.05$). Western blotting detected high expression of HSP105 in melanoma cell lines and tissues. The expression of HSP105 was related to the invasiveness of the lesions. Melanocytic naevi expressed HSP105 at a level that was similar to that of normal skin. Our results show that high expression of HSP105 is associated with malignant melanoma especially advanced and metastatic lesions. The results suggest that HSP105 analysis may be a helpful tool as a poor prognostic indicator and as a diagnostic aid in problematic lesions; in addition, melanoma can be included in the growing list of tumours overexpressing HSP105 to be targeted for potential HSP105-based therapeutic strategies. *Melanoma Res* 18:166–171 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Malignant melanoma results in the highest number of skin cancer-related deaths. Several strategies have been used in the treatment of melanoma, with no significant improvement in prognosis. It is essential to identify molecules that are highly expressed in melanoma and to target them in future treatment options. It is also important to find molecules that can aid in the differential diagnosis of malignant melanoma and benign lesions that might mimic it clinically and histologically. We report here differential expression of heat shock protein 105 (HSP105) in malignant and benign melanocytic lesions and the analysis of the relationship of this expression to the clinical characteristics of the patients.

HSP105 is a mammalian stress protein in the HSP105/HSP110 family. It was discovered from murine FM3A cells [1] and was shown by immunofluorescence to be localized in the cytoplasm and nuclei of cells under both stressed and nonstressed conditions [2]. It has been shown to be involved in malignant cell transformation by protecting tumour cells from apoptosis [3]. Mechanisms by which HSP105 suppresses apoptosis have been elucidated in HeLa cells [4].

HSP105 is expressed constitutively at very low levels in normal tissues and overexpressed in several cancers [5,6]; however, there is no report on the expression of HSP105 in melanocytic lesions. In this study, we investigated the expression of HSP105 in malignant melanoma and in melanocytic naevi by immunohistochemistry and western blot analyses.

Materials and methods

Tissue samples

Tissue specimens were obtained from patients who underwent surgery in the Department of Dermatology and Plastic and Reconstructive Surgery at Kumamoto University Hospital. Written informed consent was obtained from all participants and the study was approved by the institutional review board.

Sixty-two archival paraffin-embedded specimens of melanoma were obtained from 50 patients ranging in age from 22 to 95 years (median 71.5 years). Forty-eight per cent of the patients were male and 52% were female. The lesions consisted of 46 primary [acral lentiginous melanoma (ALM), $n=25$; lentigo maligna melanoma, $n=9$; superficial spreading melanoma (SSM), $n=7$;

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mucosal melanoma, $n = 3$; and nodular melanoma, $n = 2$] and 16 metastatic tumours (obtained from 13 patients). Fifty-two per cent of the primary lesions consisted of ALM, which is the commonest in the Japanese population. Staging was carried out according to the International Union against Cancer/American Joint Committee on Cancer Tumour Node Metastasis Classification [7]. Primary tumours varied in thickness from *in situ* to 7.20 mm. Analysis of the clinical data was carried out based on the following classification, T1 (≤ 1.00), T2 (1.01–2.00 mm), T3 (2.01–4.00 mm) and T4 (≥ 4.00 mm). Melanocytic naevi ($n = 42$) consisted of five junctional, eight intradermal, 22 compound and seven Spitz naevi, obtained from 16 male and 26 female patients ranging in age from 2 to 66 years. Frozen samples consisted of SSM ($n = 2$), ALM ($n = 2$), metastatic melanoma ($n = 1$), melanoma *in situ* ($n = 1$) and melanocytic naevi ($n = 4$).

Cell lines

Eleven melanoma cell lines: CRL1579, G361, HMV-I, SK-MEL-28, 888mel, 526mel, COLO 38, SK-MEL-19, MEWO, HM3 KO and 164 mel were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan) and Dr Y Kawakami, Keio University (Japan).

Immunohistochemistry

Four-micrometer sections were cut from paraffin-embedded archival blocks, dried at 37°C, deparaffinized in xylene and rehydrated in graded alcohols. Antigen retrieval was carried out by heating the sections in citric acid (0.01 mmol/l, pH 6) for 10 min and cooling for 60 min at room temperature. Immunohistochemical staining was performed as described previously [5]. The primary antibody, HSP105 (Santa Cruz Biotechnology, Santa Cruz, California, USA) was used at a dilution of 1 : 100 and normal rabbit immunoglobulin-G (IgG; Upstate Cell Signaling Solutions, Temecula, California, USA) was used as a nonspecific IgG control at a dilution of 1 : 100. Colour was achieved using the diamino benzidine system and counter-staining was performed using 0.5% Giemsa's solution in phosphate buffered saline. HSP105 expression was graded into –, + and + + when an average of < 25, 26–50 and > 50% of cells per high-power field were positive, respectively. The results were viewed by two authors (F.C.M. and T.K).

Table 1 Expression of HSP105 in melanoma and melanocytic naevi

Lesion	Positive cases	(%)	(%)
Primary melanoma	34/46		76
T1	7/15	47	
T2	7/7	100	
T3	10/12	83	
T4	10/12	83	
Metastatic melanoma	14/16		87.5
Melanocytic naevus	2/42		5

HSP105, heat shock protein 105; T, tumour thickness; T1, ≤ 1.00 mm; T2, 1.01–2.00 mm; T3, 2.01–4.00 mm; T4, ≥ 4.00 mm.

Table 2 Clinical data and HSP105 expression of melanoma patients

ID	Age/sex	Melanoma type	Breslow (mm)	HSP105 expression
20	65/M	Melanoma IS	0	–
16	82/F	Melanoma IS	0	+
50	50/M	Melanoma IS	0	–
48	62/F	Melanoma IS	0	–
46	78/F	Melanoma IS	0	–
15	85/F	Melanoma IS	0	+ +
40	70/M	LMM	0.2	–
22	76/F	ALM	0.4	+
17	63/F	ALM	0.4	+
8	70/F	Mucous	0.5	–
44	72/M	LMM	0.5	–
36	56/F	ALM	0.6	–
2	74/F	ALM	0.6	+ +
31	88/M	ALM	0.9	+
3	61/M	ALM	0.9	+ +
25	87/F	ALM	1	+
12	83/F	ALM	1.3	+
34	49/F	SSM	1.6	+
35	60/F	ALM	1.8	+ +
10	69/M	SSM	2	+
14	75/M	ALM	2	+ +
4	65/M	ALM	2	+ +
27	69/M	ALM	2.1	+ +
49	75/M	ALM	2.2	–
24	88/F	ALM	2.3	+
1	53/M	ALM	2.3	+ +
9	88/M	ALM	2.6	+
11	79/F	ALM	2.6	+ +
41	76/M	LMM	2.8	+ +
19	70/M	ALM	2.9	+
18	74/F	SSM	3.2	+
30	56/M	LMM	3.4	+ +
45	91/F	ALM	3.6	–
32	81/M	SSM	3.8	+
26	72/F	ALM	4.1	–
23	86/F	ALM	4.1	+
39	54/M	Mucous	4.3	–
38	22/M	ALM	4.4	+
37	47/F	NM	4.4	+ +
6	75/F	Mucous	4.4	+ +
13	50/F	SSM	4.6	+ +
33	71/M	SSM	4.7	+ +
7	57/M	ALM	5.4	+ +
42	86/M	LMM	6.7	+
28	91/M	ALM	7.2	+ +
47	49/F	NM		+ +
5	95/F	LN metastasis		+ +
21	62/F	LN metastasis		+ +
29	44/M	LN metastasis		+ +
29	44/M	Skin metastasis		+ +
43	86/F	LN metastasis		+ +
10	69/M	LN metastasis		–
11	79/F	LN metastasis		+ +
41	76/M	Skin metastasis		+ +
30	56/M	LN metastasis		+ +
32	81/M	Skin metastasis		+ +
37	47/F	LN metastasis		+
33	71/M	LN metastasis		+ +
33	71/M	Skin metastasis		–
7	57/M	LN metastasis		+
7	57/M	Skin metastasis		+ +
42	86/M	Skin metastasis		+ +

ALM, acral lentiginous melanoma; HSP105, heat shock protein 105; IS, in situ; LMM, lentigo maligna melanoma; LN, lymph node; NM, nodular melanoma; SSM, superficial spreading melanoma.

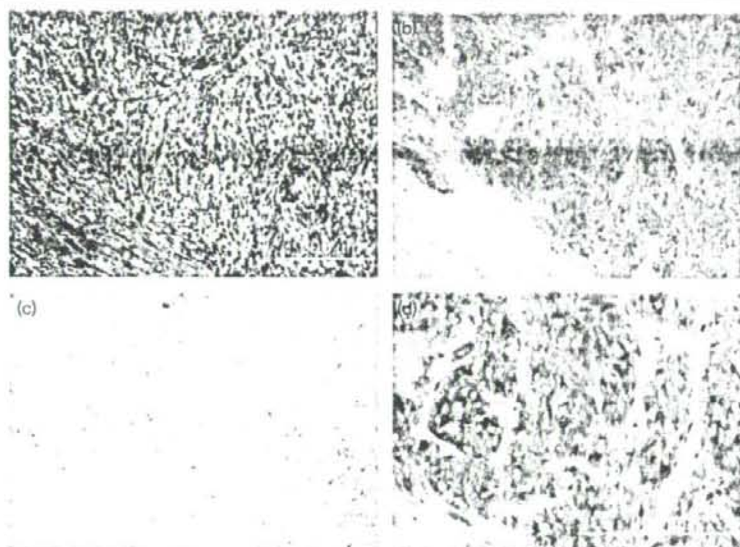
–, negative; +, positive; ++, strongly positive.

Western blotting

Tissue samples were obtained immediately after excision, snap frozen in liquid nitrogen and stored at −80°C. Primary SSM and ALM samples were classified according to the growth phase. After careful examination of the histological sections; the microinvasive, nontumorigenic parts of the tumour (radial growth phase) and the invasive tumorigenic parts (vertical growth phase) were then treated as separate sections of the same sample. Uninvolved skin from the surgical margins was also included for comparison by western blot analysis.

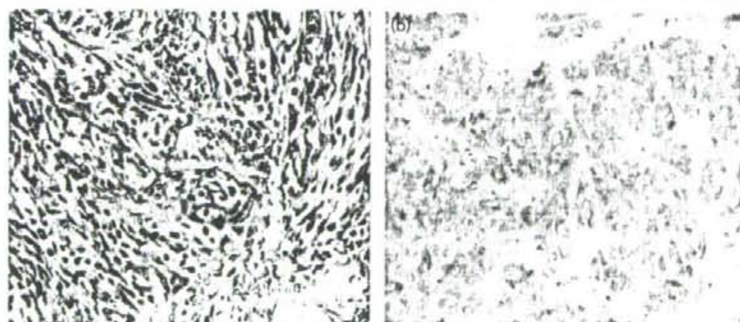
Cell lines were cultured in Dulbecco's modified Eagle's medium or Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal calf serum. The samples were homogenized and the lysate exposed to 10% SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Blocking was

Fig. 1



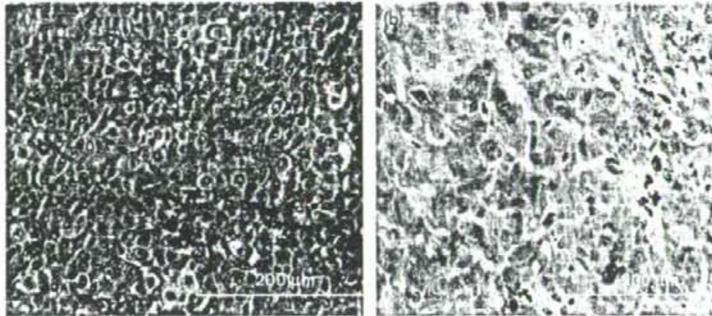
Representative immunohistochemical staining for heat shock protein 105 (HSP105) in malignant melanoma (b, d) nodular melanoma in a 47-year-old female patient, Breslow thickness of 4.4 mm (case ID 37 in Table 2). Haematoxylin and eosin stain shows the atypical, mitotically active malignant melanocytes (a). HSP105 immunohistochemical staining (b; × 100, d; × 200 original magnification, respectively) shows a nuclear and cytoplasmic staining. Normal rabbit IgG control antibody was used to eliminate nonspecific staining (c).

Fig. 2



Heat shock protein 105 immunohistochemical staining (b) of advanced acral lentiginous melanoma, thickness, 7.2 mm (case 28 in Table 2). The corresponding histological section is also shown (a). Bar 200 μm.

Fig. 3



High expression of heat shock protein 105 in metastatic melanoma lesion (b), case 29 in Table 2. The histological section is shown (a). Bar 200 μm.

achieved by incubating the membrane in 5% skimmed milk/Tris buffered saline 0.2% Twin-20 (TBST) overnight. HSP105 anti-rabbit polyclonal IgG was applied at a dilution of 1 : 500 and incubated for 60 min at room temperature, after which the membrane was incubated with goat anti-rabbit IgG-HRP (Biorad, Hercules, California, USA) for 30 min. Membranes were washed thoroughly with TBST and signals detected using the Enhanced Chemiluminescence system (Amersham Biosciences, Piscataway, New Jersey, USA). ß-actin was used as a loading control.

Statistical analysis

To compare the HSP105 expression between malignant melanoma and melanocytic naevus, the nonparametric Mann–Whitney test was performed. Samples graded –, + and + + were assigned a number (1, 2 and 3, respectively), and the two groups, melanoma and melanocytic naevus, were assigned into groups A and B, respectively. The raw data were assessed by computational analysis. The significance level was considered as P less than 0.05.

Results

Immunohistochemistry

HSP105 was highly expressed in melanoma but it showed minimal or no expression in melanocytic naevi by imunohistochemical analysis. Staining was observed in both the nuclei and cytoplasm. A total of 48 of 62 melanomas (77%) highly expressed (+ and + +) HSP105 as outlined in Table 1. Of the primary lesions, 34 of 46 (74%) overexpressed HSP105, whereas 14 of the 16 (88%) metastatic lesions were strongly positive. Increased staining was noted to be associated with an advanced stage of melanoma (Table 2); in addition, primary and metastatic lesions from the same patient maintained the same high expression of HSP105. Representative immunohistochemical staining for HSP105 in a case of nodular melanoma with a Breslow thickness of 4.4 mm, in a 47-year-old female patient, is shown in Fig. 1. Intensity of immunohistochemical staining was very high in invasive primary and metastatic lesions as shown in Figs 2 and 3. No association was,

Table 3 Clinical data and HSP105 expression of melanocytic naevi patients

ID	Age/Sex	Type	HSP105 expression
1	66/F	Junctional	–
2	10/M	Junctional	–
9	45/F	Junctional	–
17	10/M	Junctional	–
26	30/F	Junctional	–
29	33/F	Intradermal	–
31	14/M	Intradermal	–
32	35/F	Intradermal	–
13	34/F	Intradermal	–
3	39/M	Compound	–
4	5/F	Compound	–
6	5/F	Compound	–
7	28/F	Compound	–
8	33/M	Compound	–
10	15/F	Compound	–
11	9/M	Compound	–
12	32/F	Compound	–
14	54/F	Compound	–
15	44/F	Compound	–
18	9/F	Compound	–
19	10/M	Compound	–
20	2/F	Compound	–
21	11/M	Compound	–
22	4/M	Compound	–
23	4/F	Compound	+
24	20/F	Compound	–
25	25/F	Compound	–
30	20/F	Compound	–
33	9/M	Compound	–
34	29/M	Compound	–
35	2/M	Compound	–
36	20/M	Spitz	–
37	13/F	Spitz	–
38	35/M	Spitz	–
39	24/M	Spitz	–
40	3/F	Spitz	–
41	24/M	Spitz	–
42	14/F	Spitz	+ +

HSP105, heat shock protein 105; F, female; M, male.

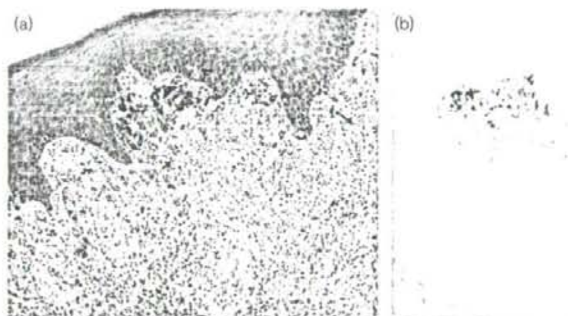
however, observed between the immunohistochemistry results and the sex or age of the patients. Only 5% (two of 42) of benign melanocytic naevi showed increased staining of HSP105 (Table 3). These consisted of a compound naevus from a 4-year-old girl and a Spitz naevus from a 14-year-old girl. No abnormality or recurrence was noted on follow-up of these cases. A representative staining of melanocytic naevus is shown in Fig. 4. A significant difference in the expression of HSP105 was observed between melanocytic naevi and malignant melanoma ($P < 0.05$).

Western blotting

Western blot analysis of four primary and one metastatic melanomas detected high expression of HSP105 in the vertical growth phase of the tumour, and much less expression in the radial growth phase of the same tumour in comparison (Fig. 5a); in addition, skin (epidermis) obtained from the surgical margins of the same patient showed a minimal expression of the protein. Melanoma *in situ* expressed HSP105 to a low level. These results are in concordance with the immunohistochemical observations, which show higher expression of HSP105 with increasing tumour thickness.

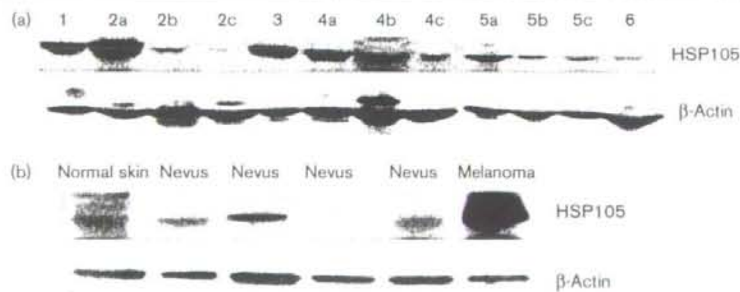
Western blotting was also performed to compare the expression of HSP105 in melanoma and melanocytic naevi. Results showed high HSP105 expression in melanoma and reduced expression in melanocytic naevi (Fig. 5b).

Fig. 4



Representative staining for melanocytic naevus, case 15 (Table 3). Haematoxylin and eosin stain shows a compound naevus with multiple naevus nests (a). Immunohistochemical staining (b) shows a negative expression of heat shock protein 105. Bar 200 μm.

Fig. 5



(a) Western blot analysis of melanoma tumours and skin from the surgical margins of the same tumour; metastatic lesions and the vertical growth phase of primary lesions show an increased expression of heat shock protein 105 (HSP105) compared with the radial growth phase and uninvolved skin from the excision margins obtained from the same patient. 1, skin metastasis; 2a, superficial spreading melanoma (SSM) vertical growth phase; 2b, SSM radial growth phase; 2c, SSM NS. 3, SSM; 4a, acral lentiginous melanoma (ALM) vertical growth phase; 4b, ALM radial growth phase; 4c, ALM NS; 5a, ALM vertical growth phase; 5b, ALM radial growth phase; 5c, ALM NS; 6, melanoma *in situ*. NS, uninvolved skin from the surgical margins. (b) Western blotting demonstrating reduced expression of HSP105 in melanocytic naevus compared with malignant melanoma. Equal loading was verified by β-Actin immunoblotting.

Eleven human melanoma cell lines, CRL1579, G361, HMV-I, SK-MEL-28, 888mel, 26mel, COLO 38, SK-MEL-19, MEWO, HM3 KO and 164mel, showed a uniformly high expression of HSP105 by western blot analysis (results not shown).

Discussion

In this study, we have, for the first time, characterized the expression of HSP105 in melanocytic lesions. We have shown an increased expression correlated with aggressive primary tumours and metastatic lesions, and markedly reduced expression in benign melanocytic naevi. Therefore, HSP105 is a useful marker and may be an unfavourable prognostic indicator in malignant melanoma. In an earlier study, we showed that HSP105 was overexpressed in squamous cell carcinoma of the skin and extramammary Paget's disease. We demonstrated a higher expression in metastatic squamous cell carcinoma compared with primary lesions. In contrast, the more indolent tumour, basal cell carcinoma, did not overexpress HSP105 [5]. A variety of other tumours have also been shown to overexpress HSP105, including colon and pancreatic adenocaricnomas, thyroid, oesophageal, breast, bladder carcinoma and others; however, the testis is the only normal tissue that overexpresses HSP105 [6].

HSP105 is a member of the heat shock family of proteins, whose functions include: acting as in-vivo chaperons of tumour-associated antigen epitopes [8], inhibition of aggregation of denatured proteins [9], assisting in protein folding and refolding of misfolded proteins, protecting cells from the cytotoxic effects of stressors that induce their transcription, and suppression of stress-induced apoptosis [4]. In the absence of overexpression of other stress proteins, HSP105 is still effective in protecting cells against potentially lethal heat exposures by inhibiting heat-induced aggregation [10]. HSP105 overexpression in cancer cells is therefore essential for their survival. Some studies have demonstrated the ability of HSP105 to prevent apoptosis by suppressing its expression using HSP105 short interfering RNA; the cancer cells underwent apoptosis [3]. Therefore, HSP105-mediated inhibition of apoptosis promotes tumorigenesis of cancer cells.

Metastatic melanoma remains resistant to therapy; therefore, it is essential to identify other molecules present in melanoma cells that can be targeted for treatment. We observed that high HSP105 expression is related to aggressive primary tumours and metastatic lesions; showing increased expression with increasing stage and thickness of melanoma. Several studies have investigated the potential use of HSP105 as an antitumor agent. In-vivo studies on HSP105/DNA vaccination resulted in a reduction of melanoma and colorectal cancer tumour bulk using BALB/c and C57Bl/6 mice models [11]. Furthermore, in a later study, HSP105-pulsed dendritic cell immunization induced an even stronger tumour rejection response in similar mice models [12,13]. Therefore, identification of tumours that overexpress HSP105 may be important for the future treatment of these tumours.

We have shown here that HSP105 is overexpressed in melanoma and is associated with advanced clinical stage and therefore may be a poor prognostic indicator. Our findings also suggest that HSP105 may have a role in the diagnosis of challenging or problematic melanocytic lesions. In addition, advanced and metastatic melanoma, which we have shown to overexpress HSP105, may be a potential target for HSP105-based treatment options.

Acknowledgements

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肝細胞癌に対するGlypican-3（GPC3）ペプチドワクチンの臨床第Ⅰ相試験

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Phase I clinical trial of glypican-3 peptide vaccine in patients with hepatocellular carcinoma

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Key words： HCC, glypican-3, peptide vaccine, Phase I, CTL

はじめに

もう治療法がないと言われ，積極的治療が受けられなくなった進行癌患者，いわゆる「癌難民」の増加が問題化している．そのような患者の多くが民間療法や健康食品などに頼っている中で，免疫療法への期待や需要は大きい．免疫療法は癌の第4の治療法として期待されているが，まだ標準治療としては確立されていないのが現状である．我々の研究は，治療法のない「癌難民」患者に有効な治療法として提供できる，科学的根拠に基づいた癌免疫療法の開発を目的としている．また一方で，免疫療法がより有効であるのは，癌の発症予防や再発予防であると考えられることから，免疫療法を用いた根治治療後の再発予防法や癌発症予防法の開発を目指している．

最近，日本国内の様々な施設から癌に対するペプチドワクチンの有効例の報告が相次いでいるが，現在，我々も国立がんセンター東病院において，進行肝細胞癌患者を対象に，肝細胞癌特異抗原glypican-3（GPC3）のペプチドワクチン臨床第Ⅰ相試験を実施している．計20例の結果からワクチンの安全性と免疫学的有効性を確認しており，約60%の患者で最低2ヶ月間は腫瘍を増悪させない効果が見られた．さらに，腫瘍マーカーの減少や腫瘍内の壊死，一部腫瘍の縮小などの臨床的な効果も認められている．

また今回の肝細胞癌の臨床試験では，事前のマウスでの研究結果と同様に，ワクチン効果に投与量依存性が示唆されたため，さらにワクチン投与量を増やす臨床試験を開始したところである．このようなワクチン療法は，もう他に治療法がない進行肝細胞癌患者にとって有用な選択肢となる可能性があるばかりでなく，手術やラジオ波焼灼療法（RFA）などの肝細胞癌根治的治療後の再発予防や，肝硬変患者に対する肝細胞癌発症予防に対しても有効であると考えており，今後はそれらに対する第Ⅱ相試験も実施する．

現在，補助療法としての抗癌剤治療は，有効な場合もあるが，無効で有害事象だけが生じる場合も少なくない．有害事象のないワクチンの補助療法としての有効性が証明できれば，標準的補助療法として確立することができ，抗癌剤治療に頼ってきた癌治療を大きく変え，患者のQOLの改善にとっても大きな役割を果たすものと考えている．また，将来的にワクチンによる癌の発症予防法や再発予防法が確立できれば，癌患者数の減少に大いに貢献できるものと考えている．

1．cDNAマイクロアレイ解析データより同定した肝細胞癌特異抗原GPC3

癌抗原の免疫療法への応用を考える場合には，発現頻度（汎用性），腫瘍特異性，免疫原性，腫瘍拒絶能，

抗原消失性，自己免疫などの副作用などによって各抗原の特徴をとらえる必要がある．理想的な癌拒絶抗原が備えているべき性質として，癌患者の体内において免疫応答を誘導する抗原であることはもちろんであるが，発現の組織特異性が優れた抗原であるということは極めて重要な要素となる．

即ち，癌細胞での発現は強いが，正常組織にはほとんど発現しておらず，腫瘍抗原に対する免疫応答が重篤な自己免疫疾患を誘導しないものとして，例えば，胎生期組織および癌細胞のみに発現する癌胎児性抗原や，癌細胞と免疫系から隔離された組織のみに発現する癌精巣抗原（Cancer-Testis抗原）などが有望である．

我々は東大医科研・ヒトゲノムセンター（中村祐輔教授）との共同研究により，cDNAマイクロアレイのデータを用いて，HCCにおける理想的な癌抗原としてふさわしいGlypican-3（GPC3）の同定に成功した[1]．まずHCC20例の癌部と非癌部における23,040種類の遺伝子の発現を比較検討することにより，多くの症例で癌部／非癌部の発現の比が5以上のものを選び，次に29臓器（胎生期の4臓器を含む）の正常臓器における23,040種類の遺伝子の発現プロフィールを解析して，胎生期の組織あるいは免疫学的に隔離された胎盤や精巣にしか発現しない遺伝子を選んだ．我々が同定したGPC3は，HCC20例中16例で癌部／非癌部の発現の比が5以上（平均14.4）で，胎盤および胎生期の肝臓，腎臓，肺に発現する以外はほとんどの正常臓器に発現を認めなかった．既に，GPC3がHCCで高発現することが報告されており，さらに我々もGPC3遺伝子および蛋白質はほとんどの肝細胞癌組織ならびに細胞株で高発現するが，正常組織においては胎生期の肝臓あるいは免疫学的に隔離された胎盤でしか発現が見られないことを実際に確認した．

新しい癌胎児性抗原GPC3は580アミノ酸からなる60kDの膜蛋白質で，HSPGs（ヘパラン硫酸プロテオグリカン）ファミリーに属する糖鎖修飾が強いGPIアンカー蛋白質であり，X染色体（Xq26）連鎖疾患で，巨人症，嚢胞腎，口蓋裂ほかの症状を呈するSimpson-Golabi-Behmel 症候群（SGBS）の患者において遺伝子変異を認める[2,3]．また，GPC3遺伝子の標的破壊マウスは，SGBSと同様に巨大化し種々の奇形を発現する[4]．HCCにおける機能についてはまだよくわかっていない．GPC3は膜蛋白で分泌され，既知のαフェトプロテイン（AFP）およびPIVKA-IIにつぐHCCの第3の腫瘍マーカーとしての有用性も示された[1]．また，我々はGPC3がメラノーマの腫瘍マーカーとしても有用であることも報告している[5～7]．

このようにGPC3の発現は腫瘍組織特異性が優れていることから，理想的な腫瘍拒絶抗原になりえるかどうかを検討した．

日本人の約60%が陽性であるHLA-A24に結合しうるGPC3由来のペプチドを合成し，これらをBALB/cマウスあるいはHLA-A24 transgenic mouse（Tgm）に免疫して解析し，HLA-A24結合性CTLエピトープペプチドEYILSLEELを同定した[8]．このペプチドEYILSLEELを用いて，ヒトのHLA-A24陽性のHCC患者の末梢血リンパ球を刺激することで，約半数からGPC3特異的CTLを誘導することができた[9]．また，日本人の40%が陽性であるHLA-A2に結合しうるペプチドを合成し，これらをHLA-A2Tgmに免疫して解析した．同定したHLA-A2結合性GPC3由来ペプチドFVGEFFTDVを用いてヒトのHLA-A2陽性のHCC患者の末梢血リンパ球を刺激することで，約半数からGPC3特異的CTLを誘導することができた[9]．これらのCTLはGPC3発現癌細胞を傷害し，免疫不全マウスの皮下に移植したGPC3発現ヒト肝癌細胞株の増殖を抑制した．また，非常に重要な点であるが，マウスを用いた以上のいずれの実験においてもGPC3抗原の免疫によってペプチド特異的CTLが誘導され，抗腫瘍効果は認められたが自己免疫現象は誘導されなかった[8～10]．

2．前臨床試験の概要

最善のプロトコール作成のため，臨床試験で用いる2種類のGPC3ペプチドと共に投与する至適アジュバントの検討を行なった[11]．BALB/cマウスを用いて，ペプチド単独群，不完全フロイントアジュバント（IFA）との併用群，CpG併用群，α-GalCel併用群，アルミニュウム併用群の5群で比較したところ，IFAとの併用投与群においてのみ，GPC3特異的なキラーT細胞（CTL）が誘導された．ペプチド単独では無効で，不完全フロイントアジュバント（IFA）と混合すると有効になることを証明し，臨床試験ではペプチドとIFAの混合物を投与することとした．次に，ペプチド投与量によって免疫応答の誘導能に相違が見られるかを検討した．BALB/cマウスにHLA-A24結合性ペプチドをIFAとともに0，5，10，20，50μg投与し比較した．その結果，CTLの誘導能はペプチド投与量に依存し，50μgの投与群で最も多くのCTLが誘導された．次に投与回数について検討した．ペプチド投与量を1.67，5，16.7，50μgの4群にわけ，それぞれの投与量において1回から4回，1週間毎に免疫した．その結果，2回

以上の免疫で投与量が16.7μg以上で，抗原特異的な免疫応答が観察された．一方で少量の投与量においては，免疫回数を増やしても同様の免疫反応は観察されなかった．さらに，HLA-A2に結合するペプチドに関してHLA-A2 Tgmを用いて検討を行ったところ，CTL誘導能は同様にペプチド投与量に依存することが観察された．

ペプチドワクチンに用いるアジュバントとして，少なくともIFAは必須であると考えられた．その他のアジュバントの併用による免疫増強効果に関しては今後の検討課題である．また，ペプチドワクチンにおけるペプチド投与量についてのこれまでのコンセンサスとして，免疫応答の強弱はペプチド量には依存しないと考えられている．しかしながら今回の我々が行ったマウスの実験では，ペプチド投与量に依存して強い免疫を誘導できるとの結果に至った．ただ，単純に体重換算すると，マウスでの50μgはヒトでの100mgに相当し，コストも膨大となるばかりか，その溶液を皮内注射するとなれば1回に100ヶ所も注射しなければならない量であり，現実的には不可能である．今回の第Ⅰ相臨床試験では，投与回数を3回，投与量を0.3，1.0，3.0mgの3段階とし，安全性を確認しながら容量を増やしていく設定にし，免疫学的モニタリングにより次相の至適投与量，投与回数を決める方針にした．

3．国立がんセンター倫理審査委員会の承認およびプロトコールの概要

動物を使った毒性試験を第3者機関に依託し，GPC3ペプチドのマウスを用いた単回皮下投与毒性試験を実施して安全性を確認した後に，進行癌患者を対象とする第Ⅰ相試験を計画し，国立がんセンター倫理審査委員会の承認を得た．平成19年2月8日，「進行肝細胞がん患者を対象としたHLA-A24および-A2結合性Glypican-3（GPC3）由来ペプチドワクチンの臨床第Ⅰ相試験」をスタートした．

プロトコールの概要を以下に示す．

1）目的

肝細胞癌には切除術をはじめとする様々な局所治療法や抗癌剤を用いた動注あるいは全身化学療法が行われているが，これらの治療に抵抗性あるいはその適応の無い患者も決して少なくない．このような病状に対する適当な治療法はなく，症状緩和など支持療法が行われているのが現状である．本研究は，局所療法または抗癌剤による化学療法が無効あるいはその適応のない，HLA-A24あるいは-A2陽性の肝細胞癌患者を対象とした，HLA-A24あるいは-A2結合性GPC3由来ペプチドワクチンを用いた免疫療法の臨床第Ⅰ相試験である．本研究はGPC3由来ペプチドワクチンの安全性を評価するとともに，本ペプチドワクチンの投与量の違いにより，末梢血中のGPC3ペプチド特異的CTLが増加するかを評価し，至適投与量を決定することを目的とする．

2）対象

局所療法または抗癌剤による化学療法が無効あるいはその適応がないと判断された肝細胞癌患者で，HLAタイピング検査によりHLA-A24あるいは-A2陽性であることが確認された患者．

3）治療

HLAのタイプにより，HLA-A24 結合性GPC3由来ペプチド（EYILSLEEL）またはHLA-A2結合性GPC3由来ペプチド（FVGEFFTDV）を用いる．2週間に1回，計3回投与する．

4）免疫学的モニタリング

①IFN-γ ELISPOT解析とHLA-GPC3ペプチド複合体ペンタマーを用いて，末梢血中のGPC3ペプチド特異的T細胞の増加程度を観察する．

②遅延型過敏症（DTH）反応を観察する．

5）主要評価項目

①HLA-A24結合性GPC3由来ペプチド（EYILSLEEL）ワクチンおよびHLA-A2結合性GPC3由来ペプチド（FVGEFFTDV）ワクチンによる有害事象の種類と発現割合．

②免疫学的モニタリングによる特異的免疫反応の誘導の観察．

6）副次評価項目

①奏効割合

奏効割合の解析対象集団を対象として3回目のワクチン終了1ヶ月後に画像診断を行い，Response Evaluation Criteria in Solid Tumors（RECIST）に従って1回目の判定をし，SD，PR，CRの症例に関しては，さらにその4週間後以降に同様の検査を行い，評価する．

②腫瘍マーカーの推移

同様に3回目のワクチン終了1ヶ月後およびその4週間後以降に3種類の腫瘍マーカー（AFP，PIVKA-II，GPC3）を測定して記録し，ペプチドワクチンの効果を，腫瘍マーカーの値の推移によっても評価する．

4．臨床第Ⅰ相試験の現時点での結果のまとめ

1）進行肝細胞癌患者を対象に，肝細胞癌特異抗原Glypican-3（GPC3）を標的とするペプチドワクチンの臨床第Ⅰ相試験を実施している．

2）投与回数を3回，投与量を0.3，1.0，3.0mgの3段階とし，安全性を確認しながら容量を増やしていく設定にした．計20例全例にDLTは発現せず，安全性に問題はないと判断された．（うち2例は明らかな病態の増悪により，ワクチン投与2回で中止し，外部委員3名からなる効果・安全性評価委員会で承認された）

3）免疫学的解析が終了した20例中19例（95.0%）に末梢血中ペプチド特異的CTLの頻度の増加が検出され，免疫学的有効性も確認された．

4）3回のワクチン投与後1ヶ月後のRECIST基準に基づく臨床効果の評価では18人中11人（61.1%）がSDで7人はPDであったが，その内訳は0.3mgの6人中3人（50.0%），1.0mgでは6人中4人（66.7%），3.0mgでは6人中4人（66.7%）がSDであり，3.0mg投与の6人中3人には腫瘍内の壊死，一部腫瘍の縮小などの所見も認められた．

5）ワクチン投与後に腫瘍マーカーの低下が見られた症例は，0.3mgの6人中0人（0%），1.0mgでは6人中3人（50%），3.0mgでは5人中5人（100%）と，我々のマウスでの研究結果と同様，臨床効果には投与量依存性も示唆された．

6）次相の至適投与量を決定するため，10.0mg，30.0mg 3回投与を3例ずつ行うこととし，倫理審査委員会に承認され，現在3例に10.0mgを3回投与した．

7）また，本臨床試験終了後生存中の患者のほとんどは，3回のペプチドワクチン投与による免疫学的効果あるいは臨床効果が認められていながら，患者の強い希望があっても，プロトコールのためにワクチンを追加投与できない状況にあった．安全性が確立した投与量で継続投与できるようプロトコールを改訂し，倫理審査委員会の承認を受け，4例に4回目として3.0mgのワクチンを投与した．うち3例は現在も継続中である．

5．今後の計画

臨床第Ⅰ相試験を終了し，GPC3由来ペプチドワクチンの安全性と免疫学的有効性を確認し，次相以降の至適投与量，投与回数を決定する．GPC3を標的とするペプチドワクチンは，他に治療法がない進行肝細胞癌患者にとって有用な選択肢になる可能性もあり，今後は第Ⅱ相試験を計画，実施する．

またこのようなワクチン療法は，元来，腫瘍がない，あるいはCTで見えない腫瘍があったとしても腫瘍量が少ない状態でこそ効果を発揮すると考えられ，手術やRFAなどの肝細胞癌根治的治療後の再発予防や，肝硬変患者に対する肝細胞癌発症予防にこそ活かされるべきであり，それらにおける第Ⅱ相試験も計画，実施する．

おわりに

癌免疫療法の多くの臨床応用の結果は，我々に失望を与えたかに見えていたが，様々な臨床試験の結果，生体の中で確かに抗腫瘍免疫応答は起きていることが証明されてきており，今回我々もまだ途中経過ではあるが，最小投与量0.3mgのペプチドワクチン投与でも末梢血中のGPC3ペプチド特異的CTLの増加を誘導できることが確認でき，それなりの臨床効果も観察できた．今後は再発予防や発癌予防に対するペプチドワクチンの効果を検証していくとともに，他の治療法と免疫療法との併用により有効な治療法を開発していきたいと考えている．

免疫療法はまだ標準治療にはなっていないが，副作用のないがん特異的免疫療法が，がんの治療・再発予防・予防法として標準化される時代はきっと来ると信じている．しかし，本当に有効な癌免疫療法を見つけるためには，膨大なスクリーニング，即ち多数の臨床試験が必要であり，様々な癌に対してどのような免疫療法がどのような対象に有効であるのか，多くの施設で様々な臨床試験を積み重ねていくことで，着実に結果を出していく必要がある．その中から本当に有効な免疫療法が開発され，標準治療の仲間入りができるよう，我々はさらに努力を積み重ねなければならない．

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