Identification of Cell Surface Antigen Expression in Canine Hepatocellular Carcinoma Cell Lines

Ayumi FUJIMOTO1, Sakurako NEO2, Chinatsu ISHIZUKA2, Takashi KATO1, Kazuhito SEGAWA1, Shinpei KAWARA1, Kikumi OGIHARA3, Masaharu HISASUE1* and Ryo TSUCHIYA1

1)Laboratory of Veterinary Internal Medicine II, School of Veterinary Medicine, Azabu University, 1–17–71 Fuchinobe, Chuoku, Sagamihara, Kanagawa 252–5201, Japan
2)Department of Veterinary Teaching Hospital, School of Life and Environmental Science, Azabu University, 1–17–71 Fuchinobe, Chuoku, Sagamihara, Kanagawa 252–5201, Japan
3)Laboratory of Pathology, School of Life and Environmental Science, Azabu University, 1–17–71 Fuchinobe, Chuoku, Sagamihara, Kanagawa 252–5201, Japan

(Received 17 December 2012/Accepted 30 January 2013/Published online in J-STAGE 13 February 2013)

ABSTRACT: The characteristics of surface antigens in canine hepatocellular carcinoma (cHCC) have not been clarified. The objective of this study was to investigate surface antigens, which are considered as stem/progenitor or cancer cell markers, in cHCC cell lines. Expression of various antigens including CD29, CD34, CD44, CD90, CD133 and Dlk-1 was assessed in four cHCC cell lines by flow cytometry. CD44, CD133 and Dlk-1 expression was detectable in all cell lines, and three cell lines expressed CD29. These results indicate that CD29, CD44, CD133 and Dlk-1 have potential as suitable markers in cHCC identification, suggesting that these findings will contribute to the establishment of an early diagnostic tool for the identification of hepatocellular maturation processes.

KEYWORDS: canine, hepatocellular carcinoma, liver, stem cell, tumor marker.


Canine hepatocellular carcinoma (cHCC) is an uncommon condition, accounting for <1% of all canine tumors. However, cHCC is the most common primary liver tumor, accounting for 50% of cases [13]. To date, no breed and sex predisposition has been confirmed for cHCC, but male dogs and dogs in general aged >10 years are over-represented in some studies [6, 9, 12]. Clinical signs of hepatic tumors are non-specific, mostly indiscernible from those of other liver diseases. Hepatic tumors are usually recognized only in the advanced stages or when metastasis has already been established. Characterization of hepatic progenitor cells (HPCs) is one of the key parameters in understanding the cellular mechanisms involved in tumorigenesis, along with the establishment of early diagnostic tools and the development of a therapeutic method. In human medicine, it is widely accepted that cancer cells arise from stem/progenitor cells, as these are the only single cells persisting in tissues for sufficient time to acquire the requisite number of genetic changes for neoplastic development [1]. Although it has been reported that various antigens expressed in liver stem/progenitor cells match with hepatocellular carcinoma (HCC) cell surface markers, including cancer stem cells, controversy continues in regard to the biological significance and specificity of various antigens in liver tissue [8]. Furthermore, identification of hepatic stem/progenitor cells is required to perform analysis of hepatocyte differentiation and maturation. Hepatic stem and/or progenitor cells not only play a pivotal role in liver regeneration but are also able to accumulate mutations in the target genes, resulting in malignant transformation [3].

In human medicine, HPCs can be more easily identified on the basis of their expression of immunohistochemical markers including CD13, CD44, CD90, CD133, epithelial cell adhesion molecule and OV6 [10]. However, research on the identification of stem/progenitor cell markers in cHCC is limited [4]. Cogliati et al. reported that CD44 and keratin 19 were expressed in most cHCC cases, while CD133 was expressed in some cases [4]. The usefulness of these markers has not been investigated, and analytical research into other HCC-specific antigens, including cancer stem cells and hepatocellular stem/progenitor cell markers, has not been conducted. Alison maintained that the rodent counterparts to HPCs described in human models are often referred to as oval cells, which express certain antigens traditionally associated with hematopoietic cells (CD34, c-kit, flt-3 and Thy-1) [1]. The information of expressing antigens in cHCC cell line may be helpful to select specific antigens in cHCC tissue, and it will contribute to the diagnosis of cHCC using immunohistochemistry of biopsy samples obtained from clinical case. In the present study, we investigated the expression level of some surface antigens recognized as tumor makers in four cHCC cell lines (930-599A, 95-0112, 95-1044 and CHKS-rL) by flow cytometry.

All tumors were removed from dogs with cHCC and diagnosed histologically. 95-0112 was established after transplantation to Rowett nude rats and cultured through >30 passages, whereas 930-599A and 95-1044 were cul-

©2013 The Japanese Society of Veterinary Science
tured through >200 passages. A CHKS-rL cell line secreting α-fetoprotein has been established and reported previously [7]. All cell lines were grown in Isocove’s modified Dulbecco’s medium (Gibco Brl, Tokyo, Japan) supplemented with 10% fetal bovine serum, 5,000 units/l penicillin, 50 mg/ml streptomycin (Gibco Brl) and 2 mmol/l L-glutamine (Sigma, St. Louis, MO, U.S.A.) in 100-mm non-coated plastic dishes at 37°C in a 5% CO2 humidified incubator. Morphological characters were as follows: 930-599A cells had spindle-shaped cytoplasm; 95-0112 cells had flattened, polygonal cytoplasm and some projections, and cell–cell adherence was not recognized; 95-1044 cells had spindle-shaped cytoplasm with tight cell–cell junctions and cell–matrix adherence; and CHKS-rL cells were of polygonal shape with strong cell–cell adherence and rounded nuclei (Fig. 1). The medium was changed every 3 days, and cells were used for flow cytometry analysis after 3–5 passages from the start of culture.

Liver tissue from 3 adult dogs was used for analysis of the mature hepatocyte population. The left lateral lobe (approximately 30 g) was surgically removed from clinically healthy 1-year-old beagles under anesthesia with atropine (0.05 mg/kg SC), propofol (8 mg/kg IV) and isoﬂuran. All experiments were conducted with humane care and in compliance with the guidelines of Azabu University for the treatment of experimental animals (100323-1). The portal branch was canulated and perfused with 300 ml of Ca2+ and Mg2+-free Hank’s buffered salt solution supplemented with HEPES (2.38 g/l) and EGTA (0.19 g/l). Tissues were then incubated at 37°C for 20 min with a perfusate of 300 ml collagenase solution ([Hanks’ buffered salt solution supplemented with HEPES (2.38 g/l) and CaCl2 (0.56 g/l)]. The digested tissue was perfused again with the addition of 300 ml serum-free Dulbecco’s modified Eagle’s medium (Gibco Brl), and the cell suspension was collected. The cells were centrifuged at 50 × g for 3 min, and the separated hepatocytes were used for flow cytometry analysis.

The samples were adjusted to a density of 1 × 10^7/ml and incubated at 4°C for 30 min with monoclonal antibodies, which were diluted ×50 in phosphate-buffered saline. Antibodies used were as follows: CD29 (PE-conjugated mouse anti-human CD29, IgG1,κ, 303004; BioLegend, San Diego, CA, U.S.A.); CD34 (PE-conjugated mouse anti-canine CD34, IgG1,κ, 559369; BD Biosciences, San Diego, CA, U.S.A.); CD44 (PE-conjugated rat anti-mouse/human CD44, IgG2b,κ, 103008; BioLegend); CD90 (Thy-1) (PE-conjugated rat anti-canine CD90, IgG2b,κ, 12-5900; eBioscience, San Diego, CA, U.S.A.); CD133 (PE-conjugated rat anti-mouse CD133, IgG1,κ, 12-1331; eBioscience) and Dlk-1 (FITC-conjugated rat anti-mouse Dlk-1, IgG1,κ, D187-4; MBL, Nagoya, Japan). Isotype-matched immunoglobulins serving as negative controls were as follows: PE-labeled mouse IgG1,κ (550617; BD Biosciences) for CD29 and CD34; PE-labeled rat IgG2b,κ (556925; BD Biosciences) for CD44 and CD90; PE-labeled rat IgG1,κ (12-4301; eBioscience) for CD133 and FITC-labeled rat IgG1,κ (M080-4; MBL) for Dlk. Samples were analyzed using an EC800 cell analyzer (TreeStar, Ashland, OR, U.S.A.). A positive result was defined as fluorescence values >10%, and high, moderate and low levels were defined as those >70, 40 and 10%, respectively. All experiments were conducted independently and in triplicate. Statistical analysis was performed using EXCEL (Microsoft) and add-in software EXCEL Toukei (Social Survey Research Information). Data regarding expression of surface antigens on mature hepatocytes are expressed as mean ± standard deviation.

All four eHCC cell lines expressed CD44, CD133 and Dlk-1 (Fig. 2 and Table 1). Marked CD29 expression was detected in 930-599A, 95-1044 and CHKS-rL (99.30, 84.43 and 94.30%, respectively), but 95-0112 did not express this antigen (1.88%). All cell lines did not express CD34, with the range of the expression level being between 0.13 and 5.04%. Markedly high CD44 expression was detected in 930-599A, 95-0112 and CHKS-rL (98.87, 78.90 and 86.63%, respectively), while a low expression level was observed in 95-1044 (29.07%). CD90 expression was detectable in 930-599A alone, but at a high level (98.57%). CD133 was moderately expressed in 930-599A (57.53%), but was expressed at low levels in 95-0112, 95-1044 and CHKS-rL (20.97, 34.67 and 19.70%, respectively). Dlk-1 was highly expressed in 930-599A (86.50%), but at low levels in 95-0112, 95-1044 and CHKS-rL (20.90, 26.43 and 21.17%, respectively). Expression of all surface antigens on mature hepatocytes of liver tissue derived from normal healthy dogs was recorded at very low levels (n=3; range, 2.16 ± 0.02 to 7.75 ± 0.09%).

In this study, CD44, CD133 and Dlk-1 were expressed in all eHCC cell lines, but not in normal hepatocytes. All cell lines showed high expression of CD44, a member of the family of transmembrane glycoproteins. This finding is relatively consistent with that of chHCC studies, indicating that CD44+ cell populations are involved as stem/progenitor cells [4, 10, 22]. One recent study indicated that CD44 is a specific marker for small or immature hepatocytes, with 61.6% of eHCC cases showing immature CD44+ hepatocytes [4]. Interestingly, expression of CD133, a surface antigen of hematopoietic stem cells, was observed in eHCC. In addition, another recent study demonstrated that hepatocellular cancer stem cells expressed CD133, and the CD133+ population had both in vitro and in vivo tumorigenic potential [11, 15]. Indeed, when Cigliati et al. investigated CD133 expression among chHCC cases, only two (15.8%) showed a high expression level [4]. We suspect that the low CD133 expression level in the present study was caused by the method of immunohistochemical analysis used, because antigen-specific reactivity decreased by less than that measured by flow cytometry. Dlk-1, a transmembrane-secreted protein with epidermal growth factor-like repeats, was identified as a hepatic stem/progenitor cell marker, and in the present study, it was expressed among all cell lines analyzed. This surface antigen was found to be associated with early liver tissue development, because it was strongly expressed in mouse fetal liver between E10.5 and E16.5 and decreased in adult liver tissue [17]. Furthermore, Yanai et al. reported that Dlk-1 was expressed in 79 of 386 cases (20.5%) of eHCC according to immunohistochemical analysis [21].
In the present study, 3 of 4 cell lines showed high CD29 expression, suggesting that this might be a progenitor surface marker for canine liver tissue. CD29 (β-integrin) is a cell surface adhesion receptor; integrin is composed of α and β subunits, which mediate the cell–extracellular matrix and cell–cell interactions. In particular, β-integrin transduces biochemical signals from the extracellular environment, mainly with respect to growth, differentiation, invasiveness and metastatic aspects of malignant cells. Suzuki et al. demonstrated that fetal mouse liver cell populations expressing CD29 and CD49f differentiated to form colonies containing mature hepatocytes and cholangiocytes, indicating that these markers have potential as hepatic stem cell markers [16]. Furthermore, we noted that CD29 was expressed in regenerative liver tissue after hepatectomy, and this marker has shown potential as a stem/progenitor cell marker (data not shown).

The presence of CD90+ cells was found to be associated with a high incidence of distant organ metastasis, suggesting that CD90 is a cancer stem cell surface antigen [20]. In this study, only cell line 930-599A strongly expressed CD90, while expression was absent in the other cell lines investigated. However, because the proportion of cancer stem cells involved in HCC is relatively low, further analysis will be required to elucidate whether CD90 is suitable for the purpose of determining chHCC stem cells. In addition, Arends et al. reported that progenitor cells derived from healthy mature canine liver expressed CD90, suggesting its potential as a progenitor cell marker [2]. Therefore, further investigation will be needed to measure CD90 expression in hepatic cancer stem cells.

CD34, a hematopoietic stem cell antigen that is also considered a stem cell marker, was not expressed among all cell lines studied, suggesting that it would not be a suitable marker in chHCC identification. Some studies reported that CD34 was found to be valuable in distinguishing HCC from metastatic neoplasms [14, 18]. However, Saad et al. reported CD34+ sinusoidal endothelial cells in 27/30 (90%) cases of human HCC, but this antigen was either not or poorly expressed in human normal liver tissue [14]. Conigliaro et al. demonstrated that murine liver stem cells are Sca1+, CD34−, CD45−, α-fetoprotein− and albumin−, suggesting that hepatic stem/progenitor cells have a non-hematopoietic origin [5]. The present study findings indicate that CD34 may not be specific to chHCC.

There is a possibility that CD29, CD44, CD133 and Dlk-1 are expressed only in situations where liver regeneration is enhanced, as α-fetoprotein [19], when it might be difficult to distinguish between tumor and normal tissue using only these antigens. In the present study, these surface antigens were not assessed in liver tissue after hepatectomy or hepatitis, and further research is required to determine appropriate cancer cell markers. The biological significance and pathogenic role of these antigens were not investigated in this study. Furthermore, the expression of surface antigens in chHCC cell lines may be transformed by abundant repeated
passage in vitro. In particular, transplantation experiment and characterization of cell populations established using these antigens are expected to clarify the biological significance, and investigation of gene m-RNA/protein expression of other oncogenes, transcription factors and factors associated with hepatocyte differentiation/maturation will be required.

In conclusion, CD29, CD44, CD133 and Dlk-1 were expressed in cHCC cell lines, suggesting that these surface markers have potential as diagnostic tools and will be helpful in cHCC characterization. In addition, they have the potential to detect hepatocellular stem/progenitor cells in canine, similar to those in humans. We expect that evaluation of expression of these markers in biopsy samples may be helpful in establishing cHCC diagnosis. However, to determine the usefulness of this diagnostic tool in veterinary clinical pathology, the sensitivity and specificity of these antigens should be investigated among cHCC, hepatitis and hyperplastic tissues from clinical cases.

ACKNOWLEDGMENTS. This study was supported by the Science Research Promotion Fund of the Promotion and Mutual Aid Corporation for Private Schools of Japan and a Grant-in-Aid for Matching Fund Subsidy for Private Universities.
REFERENCES


