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CD44 EXPRESSION AND GROWTH FACTORS

Jun-ichi Hamada[1], Yutaka Sawamura[2], and Erwin G. Van Meir[3]

[1] Division of Cell Biology, Cancer Institute and [2] Department of Neurosurgery, Hokkaido University School of Medicine, Sapporo, Japan, [3] Laboratory of Molecular Neuro-Oncology, Neurosurgery Department and Winship Cancer Center, Emory University, Atlanta, Georgia, 30322, U.S.A. and [3] Laboratory of Tumor Biology and Genetics, Neurosurgery Department, University Hospital, 1011 Lausanne, Switzerland

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1. ABSTRACT

Soluble factors such as growth factors and cytokines present in the tumor microenvironment regulate a variety of genes associated with malignant properties of tumor cells such as growth, migration, invasion, and metastatic capacities. CD44 is a multi-functional adhesion molecule involved in cell to cell and cell to extracellular matrix interaction, the trapping of growth factors and cytokines, and the regulation of cell traffic. Growth factors and cytokines modify the expression, selective isoform splicing and functions of CD44, resulting in changes in the biological properties of the cells. These include adhesion of circulating tumor cells to endothelium and body cavities, and survival in response to growth factors presented by the CD44 molecule. The modification of CD44 on both tumor and host cells by growth factors may play an important role in tumor progression.

2. INTRODUCTION

Tumor progression is the process by which tumor cells acquire malignant properties, such as progressive growth, invasion, and metastasis (1). Adhesion molecules are involved in tumor progression in various ways. Some adhesion molecules such as cadherins, connexins, and alpha-4-beta-1 integrin support homeostasis through the maintenance of cell to cell contact. Other types of adhesion molecules such as CD44, and those which belong to the integrin and immunoglobulin superfamilies, are necessary for migration, invasion, and metastasis. In short, adhesion molecules serve as both suppressors and promoters in carcinogenesis and the subsequent progression to malignancy. CD44 is considered to be one of the most important adhesion molecules which facilitate tumor invasion and metastasis (2,3). This molecule functions not only as a receptor for cell to cell or cell to matrix adhesion, but also as a signal transmitter and growth factor-presenting molecule.

Tumors are influenced by their microenvironment. This includes normal cells, extracellular matrices and soluble factors such as growth factors, cytokines and hormones (4,5,6). Growth factors and cytokines in particular have been shown to enhance the invasiveness, motility, and growth of tumor cells *in vitro*. Therefore, this review discusses the influence of growth factors and cytokines on the expression and functions of CD44, and, in turn, the relationship of this influence upon malignant progression.

3. ALTERATION OF CD44 EXPRESSION BY THE STIMULATION WITH GROWTH FACTORS AND CYTOKINES

Modification of the expression and functions of CD44 by growth factors or cytokines is classified into four processes: 1) the increase or decrease in the expression of CD44 isoforms which are expressed constitutively; 2) the novel induction of CD44 isoforms; 3) the alteration of the glycosylation of CD44; and 4) the activation of CD44 function. Growth factors and cytokines which modify CD44 expression and functions are shown in table 1; note that many of them are growth factors and cytokines produced in the process of acute or chronic inflammation which upregulate CD44 expression. Upregulation of CD44 by inflammatory cytokines may imply that inflammation is one of the factors that enhance malignant progression of tumors.

In many types of cancer, including breast cancer, ovarian cancer, and head and neck cancer, tumor cells frequently express abnormal or amplified growth-factor receptors, one of which is the $p185^{HER2/neu}$, *HER2* oncogene (also called c-*erbB2* or *neu*) product. Recently, overexpression of $p185^{HER2/neu}$, which is a constitutively-

Table 1. Modification of CD44 expression and functions by growth factors and cytokines

TARGET CELLS	GROWTH FACTORS/CYTOKINES	CD44 EXPRESSION	REFERENCE
TUMOR CELLS			
Human neuroblastoma (SK-N-SH)	IGF-1 PDGF	Increase (CD44v3, v5, v6, v7, v8)	12
	NGF	Decrease (CD44v3, v6, v9)	12
Human renal cell carcinoma (R11)	IL-2 IFN-alpha	Decrease	50
Human renal cell carcinoma (CCF-RC7)	IL-1beta IFN-gamma TNF-alpha	Increase	51
Human lung carcinoma (LCLC97) Human colon carcinoma (HT29) Human keratinocyte (HPKII)	IFN-gamma	Increase (CD44v6) Decrease (CD44s, v9)	13
Rat mammary carcinoma (ER-1)	TGF-beta	Increase	This reference
ENDOTHELIAL CELLS			
Human vascular endothelial cells (ECV304)	HGF	Increase	11
Human umbilical vien endothelial cell	bFGF VEGF	Increase	52
Human endothelial cell	TNF-alpha	Increase	63
FIBROBLASTS			
Mouse fibroblast (NR6-WT)	EGF	Increase	21
Human lung fibroblast (HLF)	TGF-beta bFGF	Increase in the length of the chondroitin sulfate chains	15
Mouse fibroblast (Balb/c 3T3)	PDGF	Increase	54
OTHERS			
CD34+ human hematopoietic progenitor cell	SCF GM-CSF IL-3	Enhancement of CD-44 mediates adhesion	16
Myelomonocytic cell (THP-1, U937)	TNF-alpha IFN-gamma	Increase	13
Bovine articular chondrocyte	28 kDa-FN fragment IL-1 alpha	Increase	55

IGF: Insulin-like growth factor, PDGF: Platelet derived growth factor, NGF: Nerve growth factor, IL: Interleukin, IFN: Interferon, TNF: Tumor necrosis factor, HGF: Hepatocyte growth factor, bFGF: basic fibroblast growth factor, VEGF: Vascular endothelial growth factor, EGF: Epidermal growth factor, TGF: Transforming growth factor, FN: Fibronectin, SCF: Stem cell factor, GM-CSF: Granulocyte, macrophage-colony stimulating factor.

active tyrosine-kinase receptor was found to upregulate CD44 in human ovarian cancer cells and NIH3T3 cells (7,8). Overexpression of CD44 in $p185^{HER2/neu}$-transfected NIH3T3 cells results in a dramatic enhancement of hyaluronan-mediated cell adhesion. Furthermore, it is shown that CD44 and $p185^{HER2/neu}$ are physically linked to each other in human ovarian cancer cells via interchain disulfide bonds. Hyaluronic acid can then stimulate CD44-associated $p185^{HER2}$ tyrosine kinase activity, resulting in an increase in cell growth. These results suggest that tumor cells with constitutively-active receptors like $p185^{HER2/neu}$ persistently upregulate CD44 without the stimulation of ligands, and that direct cross-talk between growth factor receptors and CD44 may be one of the most important signaling events in tumor malignancy.

The overexpression of CD44 by tumor cells may be conducive to metastasis since this adhesion molecule is thought to support the formation of homotypic aggregates of tumor cells or heterotypic aggregates of tumor cells and blood cells. These aggregates are known to arrest in the microcirculation at much higher rates than non-aggregates (9,10). Moreover, growth factors modify CD44 expression and the functions of host cells as well as tumor cells, as shown in table 1. On endothelial cells, which are one of the key determinants in the establishment of blood-borne metastasis, CD44 expression is augmented by some growth factors. Through their *in vitro* analyses of the adhesiveness of human colon or breast cancer cells to human lung vascular endothelial cells, Hiscox and Jiang have demonstrated that HGF stimulates the expression of CD44 by endothelial cells (11). Tumor cells adhere to the endothelium via CD44 expressed on the surface of endothelial cells. Although it is not clear which are the binding partners for CD44 on the tumor cells, this evidence suggests that HGF plays a key role in the augmentation of adhesiveness between endothelium and metastatic tumor cells, resulting in enhanced metastasis.

The CD44 gene contains 20 exons and generates multiple CD44 isoforms by alternative splicing. In some cases, growth factor-stimulation influences the alternative splicing patterns of CD44 nuclear RNA. For example,

human neuroblastoma cell line, SK-N-SH, which intrinsically expresses mRNA with a sequence encoded by standard exons plus variant exons v3,v6,v8, and v9, has mRNA transcripts carrying the alternative exons v3,v5, v6, v7, v8, and v9 without changes in the total mRNA levels for CD44 when treated with IGF-I or PDGF (12). On the other hand, NGF-stimulation leads to reduced levels of CD44v3, v6, and v9 mRNAs in the same cell line. These findings suggest that differential splicing of the CD44 gene may be regulated by different intracellular signal transduction mechanisms after activation of growth factor receptors. Analyses of the expression of CD44 variant isoforms in human cell lines by the use of antibodies against variant regions are also interesting (13). In myelomonocytic leukemia cell lines, both TNF-alpha and IFN-gamma modulate CD44v9 and v6 expression. In large cell lung cancer, colon cancer, and keratinocyte cell lines, IFN-gamma upregulates the expression of CD44v6 and down-regulates that of CD44s and v9. Understanding the effect that growth factors have on the splicing patterns of CD44 nuclear RNA is thus important for both the mechanisms by which malignancies progress, and the elucidation of the basic mechanisms responsible for alternative splicing.

Carbohydrate modifications on the CD44 molecule influence its functions (14). In particular, the binding affinity of the molecule for hyaluronan, a major ligand for CD44, and the ability of CD44 to trap heparin-binding growth factors are closely related to its glycosylation. There is a report stating that TGF-beta1 and bFGF affect the structure and potential interactions of the chondroitin sulfate proteoglycans on CD44 molecules in human lung fibroblasts (15). While these growth factors increase the length and number of chondroitin sulfate chains of CD44, the growth factors do not increase the levels of the protein nor the fraction processed into proteoglycan. CD44 with increased chondroitin sulfate proteoglycans shows higher affinity for fibronectin compared with CD44 derived from fibroblasts treated without TGF-beta1 or bFGF. Thus, some growth factors may modify CD44 functions by affecting glycosylation pathways.

Growth factors and cytokines also induce the activation of CD44 molecules. Legras *et al*. have investigated adhesive interactions between $CD34^+$ hematopoietic progenitor cells (HPC) and bone marrow stroma via CD44 (16). They have found that CD44-mediated adhesion of $CD34^+$ cells to hyaluronan is enhanced by an anti-CD-44 activating CD44 function or by cytokines such as GM-CSF, IL-3, and stem-cell factor. The enhanced adhesion of $CD34^+$ HPC by anti-CD44 antibody or cytokines is induced rapidly without alteration of CD44 expression.

Although little is known about the modification of the expression and functions of CD44 by growth factors and cytokines, it is likely that these factors contribute to facilitate tumor invasion and metastasis through alteration of CD44 properties.

4. SIGNAL TRANSDUCTION PATHWAYS INVOLVED IN THE UPREGULATION OF CD44 BY GROWTH FACTOR-STIMULATION

The activation of the ras and src oncogenes induces CD44 expression and alters the adhesion properties of fibroblasts and epithelial cells (17-19). The products of these oncogenes serve as intracellular signal transducers. In particular, $p21^{ras}$ is activated by tyrosine kinase-type growth factor receptors and transduces the signals to downstream effectors. Hofmann *et al*. have shown that $p21^{ras}$ promotes transcription of the CD44 gene via the AP-1 binding site in the CD44 promoter region (17). v-Fos-transformed rat fibroblasts invade a reconstituted basement membrane, Matrigel, in an AP-1-mediated manner (20). CD44 expression also increases in these cells. Considered together, these findings suggest that for regulation of CD44 expression there is a signal transduction pathway connecting stimulated growth factor receptors to a transcription factor, AP-1, through activation of $p21^{ras}$.

Recently, it was reported that CD44 gene expression induced by EGF-stimulation is mediated through an interaction between a novel 22-bp EGF regulatory element and a putative novel transcription factor in mouse fibroblasts (21). Fichter *et al*. have demonstrated that phosphoinositide 3-kinase (PI3-kinase) and TPA-sensitive protein kinase C (PKC) serve as signal transducers in the expression of CD44 in human neuroblastoma cells (12). Wortmannin, a specific inhibitor of PI3-kinase partly reduces IGF-I and PDGF capacity to induce CD44 expression in neuroblastoma cells. GF-109203X, an inhibitor of protein kinase-C, blocks CD44-upregulation by TPA- and IGF-I-stimulation.

These findings suggest that CD44 expression may be regulated by distinct signal transduction pathways involving $p21^{ras}$, PKC, and PI3-kinase. Abnormal activation of these signal transduction mediators may convert tumor cells into more malignant ones through the upregulation of CD44.

5. PRESENTATION OF GROWTH FACTORS AND CYTOKINES BY CD44

Since certain cytokines bind to CD44 expressed on endothelial cells, there is a possibility that CD44 molecules may present cytokines to blood-borne tumor cells which might promote their adhesion to endothelium and subsequent extravasation. Tanaka *et al*. have reported the role of macrophage inflammatory protein-1beta (MIP-1beta) in T-cell adhesion (22). Their results show that 1) MIP-1beta is present on endothelium; 2) immobilized MIP-1beta induces binding of T-cells to VCAM *in vitro*; and 3) MIP-1beta is immobilized by binding to CD44. Circulating T-cells recognize adhesion molecules such as selectins expressed on endothelial cells and loosely attach to them. Subsequently, T-cells are activated through binding to MIP-1beta immobilized by CD44, and then they tightly adhere and migrate to extravasate. Although, so far, there is no evidence indicating that tumor cells recognize

cytokines presented by CD44, it can be speculated that similar events might happen in the interaction between tumor cells and endothelial cells.

CD44 is modified with heparan sulfate. Growth factors and cytokines which have affinity to heparin are potent binders of heparan sulfate. Heparin-binding growth factors and cytokines comprise many factors including bFGF, HB-EGF, HGF, PDGF, and IL-8, in addition to MIP-1beta. Actually, CD44 isoforms containing the alternatively spliced exon v3 in keratinocytes bind bFGF and HB-EGF via heparan sulfate (23). Since some of the heparin-binding growth factors stimulate tumor cell growth, motility, and invasion, endothelial cells presenting growth factors on CD44 may offer favorable conditions for the survival, growth, and extravasation of tumor cells. Conversely, binding of bFGF, VEGF and IL-8 on CD44 heparan sulfate chains may stimulate endothelial cell angiogenesis.

6. INFLUENCE OF GROWTH FACTORS AND CYTOKINES ON THE PRODUCTION OF HYALURONAN, A PRINCIPAL LIGAND OF CD44

In general, molecules acting as ligands are necessary for the induction of biological responses via CD44 in tumor cells. Hyaluronic acid, which is a large glycosaminoglycan with a molecular weight ranging from one hundred thousand to several million, is the principal but not the only ligand of CD44 (24,25), and is ubiquitously present in connective tissues. It is also known that hyaluronate is present in large amounts in several types of tumors such as mammary, hepatic, lung, and parotid gland tumors. Hyaluronate is also a major component of the brain extracellular matrix. There are reports indicating that a hyaluronan-rich matrix offers a suitable microenvironment for tumor cell growth and invasion (26-31).

In vitro investigations using a coculture system of tumor cells with normal cells revealed that tumor cells stimulate normal cells to promote the production of hyaluronan (27,32). In tumor tissues, tumor cells possibly produce the mediators which stimulate hyaluronan production by normal cells. In fact, several factors are known to promote hyaluronan production by fibroblasts or mesothelial cells (**t**able 2). It is noteworthy that many of them are identical to the factors which stimulate CD44 expression and function. There is a possibility that the same growth factors act in both tumor cells and normal cells in an autocrine and a paracrine fashion to make a suitable microenvironment for invasion and metastasis by stimulating the expression of CD44 and its ligand hyaluronan.

The factors stimulating the production of hyaluronan, e.g., TGF-beta, does not always stimulate fibroblasts to promote hyaluronan synthesis. The fibroblasts show dual responses to TGF-beta (33). TGF-beta promotes hyaluronan synthesis when the fibroblasts are cultured on plastic substratum, whereas it reduces synthesis when they are on a substratum coated with collagen.

Hyaluronan is also found in clinical materials. The hyaluronan-producing activity present in the pleural fluids from mesothelioma patients, medium conditioned with mesothelioma cells and serum from breast cancer patients, may be particularly useful for the diagnosis of these cancers (34-36).

7. THE ROLES OF CD44 IN PERITONEAL DISSEMINATION

As mentioned above, the evidence that some growth factors stimulate hyaluronan production by mesothelial cells, and that reactive mesothelium expresses CD44, leads us to consider the importance of CD44 in peritoneal or pleural metastasis of cancer (34,35,37-40). This section considers the roles of CD44 in adhesion of tumor cells to mesothelium in peritoneal dissemination.

Some *in vitro* studies have demonstrated that tumor cells use CD44 molecules to attach to mesothelial cells. Cannistra *et al*. have shown that CD44-positive ovarian cancer cells exhibit significant mesothelial binding which is partly blocked by anti-CD44 antibodies (41). Pretreatment of mesothelium with hyaluronidase also inhibits the binding of ovarian tumor cells to mesothelium, suggesting that tumor CD44 recognizes hyaluronate on mesothelium. A series of reports by Turner and his colleagues also indicate that ovarian cancer cells adhere to hyaluronic acid on mesothelial cells via CD44 (42-44).

CD44 is also involved in the peritoneal dissemination of gastric cancer cells. Human scirrhous gastric cancer cells, which have a high potential to form peritoneal dissemination in nude mice, express higher levels of CD44 than cells which do not cause peritoneal dissemination (45). The binding ability of these highly metastatic cells to mesothelial cells or hyaluronic acid is partly inhibited by treatments with anti-CD44 antibodies or hyaluronidase. The *in vivo* inoculation of highly metastatic cells treated with anti-CD44 antibodies results in a significant prolongation of survival time as compared to control mice inoculated with non-treated tumor cells. These observations suggest that CD44 facilitates peritoneal dissemination of this gastric cancer cell line. In an experiment using other human gastric cancer cell lines, the treatment with antibodies to both CD44 and beta1 integrin inhibits the dissemination of gastric cancer cells in the peritoneal cavity of nude mice and prolongs their survival time (39). Further, the report shows that TGF-beta1 increases the expression of CD44 in both the tumor cells and in mesothelial cells, which results in the enhancement of adhesion and invasion to the mesothelial cell monolayer. Thus, these findings suggest that CD44 is one of the mediators involved in the attachment of gastric cancer cells to mesothelial cells and that TGF-beta1 may participate in the promotion of the dissemination.

Table 2. Modification of hyaluronan production by growth factors and cytokines

TARGET CELLS	GROWTH FACTORS/CYTOKINES	HYALURONAN PRODUCTION	REFERENCE
MESOTHELIAL CELLS			
	PDGF-BB	Increase	37
	IGF-1+EGF	Increase	38
	IL-1 beta	Increase	39
	Human mesothelioma cell-conditioned medium (heat stable, partially trypsin resistant)	Increase	34
	Pleural fluids from mesothelioma patients		35
FIBROBLASTS			
	PDGF-BB EGF bFGF TGF-beta	Increase	56
	TGF-beta PDGF-AA PDGF-BB	Increase	57
	TGF-beta	Increase	58
On a plastic substratum	TGF-beta	Increase	33
On a collagen substratum	TGF-beta	Decrease	33
	Human mesothelioma cell-conditioned medium (heat stable, partially trypsin resistant)	Increase	34
	Human lung carcinoma cell membrane fraction (heat and trypsin-sensitive	Increase	59,60
FIBROSARCOMA CELLS			
	Breast cancer patient serum (~150 kDa glycoprotein)	Increase	36

PDGF: Platelet-derived growth factor, IGF: Insulin-like growth factor, EGF: Epidermal growth factor, IL-1: Interleukin-1, bFGF: basic fibroblast growth factor, TGF: Transforming growth factor

In vivo observations strengthen the importance of the interaction of tumor cells with mesothelial cells via tumor expressed CD44 (47). When mouse ovarian or mammary ascites tumor cells are injected intra-peritoneally into mice, hyaluronan accumulates at the initial site of attachment of tumor cells and cells clump to the mesenteric surface. Immunohistochemistry using anti-CD44 antibodies reveals that the great majority of the cells that initially attach to the mesentery are strongly CD44-positive. These histopathological findings suggest the involvement of hyaluronan-rich matrix in tumor cell attachment to the mesentery through interaction with tumor cell CD44.

A weakly malignant cloned cell line, ER-1, has been derived from a rat mammary carcinoma cell line in order to study factors mediating tumor progression (48). It was found that growth factors such as EGF and TGF-beta enhance the malignancy of the ER-1 cells, which is assessed by their ability to achieve peritoneal dissemination (49). Both TGF-beta and EGF enhance the *in vitro* invasion of mesothelial cell monolayers by ER-1 cells (table 3). However, ER-1 cells treated with TGF-beta but not EGF adhere to cultured mesothelial cells or hyaluronic acid-coated plates at a higher rate compared to non-treated ER-1 cells. Analyses by flow cytometry using anti-rat CD44 antibodies revealed the increased expression levels of the stimulation with TGF-beta but not EGF (figure 1). These findings suggest that enhanced invasiveness of ER-1 cells to mesothelial cell monolayers by TGF-beta or EGF is induced by distinct mechanisms. TGF-beta-induced, but not EGF-induced, invasiveness is probably related to the upregulation of CD44 expression.

Considered together, these findings indicate that CD44 may play a key role in tumor dissemination in body cavities. Strategies to directly block CD44 function or eliminate microenvironmental factors which upregulate expression and function of CD44 and its ligand hyaluronan may result in the decreased spread of tumor cells exfoliated into body cavities.

Table 3. Enhanced effects of EGF or TGF-beta on tumorigenicity and *in vitro* invasion of mesothelial cell monolayers by rat ER-1 mammary carcinoma cells

CYTOKINE[a]	AMOUNT (ng/ml)	NUMBER OF PENETRATED COLONIES/CM^2±SD[b]	NUMBER OF RATS WITH TUMOR/NUMBER OF RATS USED[c]
EGF	0	2.3±1.1	0/5
	1	35.7±4.5	2/5
	10	95.7±8.9	5/5
	100	103.3±10.4	5/5
TGF-beta	0	2.3±1.1	0/5
	0.1	5.6±2.3	1/5
	1	38.3±4.1	4/5
	10	63.9±5.3	5/5

[a]: ER-1 cells were treated in vitro with EGF or TGF-beta for 24 hours. [b]: ER-1 cells were seeded on the rat mesothelial cell monolayer. Five days after the tumor cell seeding, the number of colonies per 1 cm^2 formed under the mesothelial cell monolayer were counted under a phase contrast microscope. [c]: ER-1 cells (1x10^5/rat) were injected intraperitoneally into syngenetic rats. Three weeks later, the rats were killed for examination of tumor formation in the peritoneum (peritoneal dissemination).

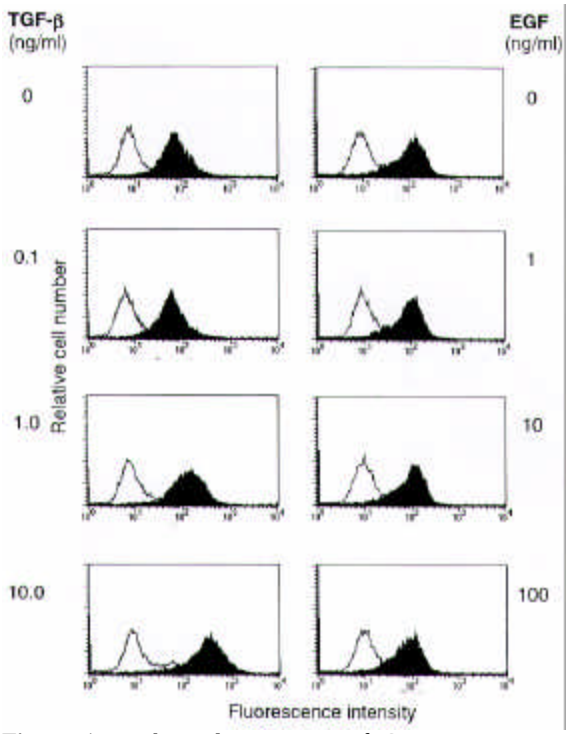

Figure 1. Enhanced expression of CD44 on rat ER-1 mammary carcinoma cells after treatment with TGF-beta. ER-1 cells were treated *in vitro* with TGF-beta (left) or EGF (right) for 24 h. CD44 expression was measured by flow cytometry using anti-rat CD44 antibodies. TGF-beta but not EGF enhances the CD44 expression on ER-1 cells in a concentration-dependent manner.

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Send correspondence to: Dr J Hamada, Division of cell Biology, Cancer Institute, Hokkaido University School of Medicine, Kita-15, Nishi-7, Kita-ku, Sapporo 060-8638 Japan, Tel:+81-11-706-5070, Fax: +81-11-706-7870, E-mail: jhamada@med.hokudai.ac.jp