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**Novel CD9 targeted therapies in gastric cancer**

Murayama Y *et al.* Manipulation of CD9 in gastric cancer

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**Abstract**

There are 33 human tetraspanin proteins, emerging as key players in malignancy, the immune system, fertilization, cellular signaling, adhesion, morphology, motility, proliferation, and tumor invasion. CD9, a member of the tetraspanin family, associates with and influences, a variety of cell surface molecules. Through these interactions, CD9 modifies multiple cellular events, including adhesion, migration, proliferation, and survival. CD9 has therefore been considered to play a role in several stages during cancer development. Indeed, the reduced CD9 expression is generally related to venous vessel invasion and metastasis as well as poor prognosis. Our reports stated that the administration of mice bearing human gastric cancer cells with anti-CD9 antibody successfully inhibited tumor progression *via* anti-proliferative, pro-apoptotic, and anti-angiogenetic effect, strongly indicated that CD9 is a possible target to treat patients with gastric cancer. Here, we will describe the possibility of CD9 manipulation as a novel therapeutic strategy in gastric cancer, which still shows poor prognosis.

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**Key words:** CD9; Tetraspanin; Gastric cancer; Tumorigenicity; Target therapies

**Core tip:** Tetraspanin CD9 is a cell surface protein with four transmembrane domains and found in multiple organs. Although CD9 was primarily identified as a tumor suppressor, it exhibits diverse functions through the association with various partner proteins. CD9 relates to tumor proliferation, apoptosis, migration, adhesion, and angiogenesis, therefore involving in several steps of tumor formation; communication with the environment, dissemination, and metastasis. In this review, we describe the possibility of CD9 manipulation as a novel therapeutic strategy to improve clinical outcome ingastric cancer.

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**INTRODUCTION**

Gastric cancer is one of the most common malignancies, remaining a major public health issue as the fourth most common cancer and the second leading cause of cancer death in the world[1], with a particularly high incidence in Japan, China, South Korea, Chile and Costa Rica. The large regional incidence variations possibly reflect different prevalences of *Helicobacter pylori* infection, which is responsible for more than 60% of gastric cancer globally. Advanced gastric cancer is a very aggressive disease, and the prognosis remains poor. The 5-year survival rate for loco-regional disease is 25%–35%[2-4] and the median survival ranges from 10 to 14 mo in advanced disease[5,6]. Although various treatment modalities have been developed and the mortality rate of gastric cancer has gradually decreased over the past several decades[7], many of them failed to eliminate gastric cancer cells curatively[8]. Therefore, a novel therapeutic strategy is clinically desired.

CD9, a member of the tetraspanin family, has been reported to relate to growth and invasion of tumor cells. There are many reports of the relationship between CD9 expression and disease prognosis. In addition, molecular mechanisms of CD9 functions have been gradually clarified. In this field, we also reported apoptotic signals after CD9 ligation in gastric cancer cells as well as the treatment of gastric cancer-bearing mice with anti-CD9 antibody.

We will review characteristics of CD9 and discuss the possibility of CD9 as a novel therapeutic target in gastric cancer.

**CD9 FUNCTIONS**

Tetraspanins, which have four putative membrane-spanning domains, are integral membrane proteins including at least 33 distinct family members, such as CD9,CD37, CD53, CD63, CD81, CD82, and CD151[9-11]. Members of this family are involved in many physiological and pathological processes, such as fertilization, cellular adhesion, motility, and tumor invasion[9-12]. To date, tetraspanins are believed to act as molecular facilitators or adaptors, which form a network of interaction among the cell surface molecules, known as the “tetraspanin web” or tetraspan-enriched microdomains[12,13]. Notably, some tetraspanin proteins have key roles in tumor initiation, promotion, metastasis, and angiogenesis.

CD9, which was identified as a suppressor of cancer spreading[14], belongs to the tetraspanin family. Like other tetraspanins, CD9 has four putative transmembrane domains, which provide the short N- and C-terminal cytoplasmic domains, a small intracellular loop, and two extracellular loops [11,12] (Figure 1). CD9 is widely expressed on the surface of several types of cells, including many malignant tumor cells as well as normal haemopoietic, endothelial, and epithelial cells[11,12].

CD9 interacts with a number of transmembrane proteins, including integrins, EWI proteins (EWI-2 and EWI-F) and other tetraspanins (*e.g*., CD81 and CD151)[10-13], Claudin-1[15], epidermal growth factor receptor (EGFR)[16], and membrane-bound ligands for EGFR[17-19](Table 1). These interactions form functional complexes, which facilitate cell adhesion, motility, and signaling[10,20-24]. For examples, antibody (Ab) ligation of CD9 induces homotypic aggregation of pre-B cells and augments their adhesion to bone marrow fibroblasts through the modification of integrins[10]. Treatment with anti-CD9 Ab can induce strong adhesion between stromal and hematopoietic cells[25,26] as well as inhibit the migration of malignant cells[27]. In addition, CD9 acts as a co-receptor for diphtheria toxin. CD9 does not bind directly to the toxin, but interacts with the diphtheria toxin receptor (transmembrane precursor of heparin-binding epidermal-growth-factor-like growth factor; HB-EGF), leading to the elevation of juxtacrine activity of HB-EGF[28,29]. Also, CD9 functionally associates with Fcγ receptors, and co-cross-linking of CD9-Fcγ receptors modifies signals for phagocytosis and inflammatory responses on macrophages[30].

CD9 affects physical processes, such as cell proliferation, apoptosis and tumor metastasis[31-33]. Treatment of cells with anti-CD9 Ab revealed anti-proliferative effects[16,18] *via* the suppression of extracellular-signal-regulated kinase 1/2 (ERK 1/2) activity[31]. In addition, CD9 ligation concurrently induced apoptosis *via* the selective activation of the c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 mitogen-activated-protein kinase (MAPK) pathway as well as caspase-3 and the p46 Shc isoform[31]. Moreover, CD9 can associate with conventional protein kinase C (PKC) isoforms including PKC α and PKC β[34], as well as type II phosphatidylinositol 4-kinase (PI4K)[35], which could contribute to tumour-suppressor functions. In addition, CD9 may affect the Wnt signalling pathway by downregulating Wnt genes[36]. Expression of CD9 also act to protect TGFαfrom cleavage, thereby regulating cell proliferation and cell migration[19]. Therefore, CD9 expression has an ability to regulate a variety of intra-cellular signals.

**CD9 AND CANCERS**

From experiments manipulating CD9 in tumor cell lines, CD9 has been demonstrated to be primarily a suppressor of metastasis[27,37-40]. Several clinical studies have also shown an important prognostic value of CD9. The reduced CD9 expression is associated with a poor prognosis in melanoma[41], non-small-cell lung cancers[28], breast cancers[37,42], colon cancers[44], pancreatic cancers[44], ovarian cancers[45], and prostate cancers[46]. The expression of CD9 is also related to metastasis of the gastrointestinal carcinoma[43,44,47,48]. For example, the reduced CD9 expression is significantly associated with more venous vessel invasion and liver metastasis in patients with colon cancers[27,44]. Although diverse physiological functions (clinical data) of CD9 have been suggested[49,50], we and others found that the amount of CD9 is inversely correlated to the lymph node status in gastric cancer[48] and in esophageal squamous cell carcinoma[47]. Moreover, expression of CD9 protein was found significantly stronger in pN0, pM0 than in advanced pN stages, pM1, respectively in gastric cancer[51]. Furthermore, the reduction of CD9 protein was associated with distant metastasis of gastric cancer. Thus, decreased levels of CD9 are strongly associated with an increased risk of recurrence, especially in patients with NO nodal status and M0 metastatic status. The low levels of CD9 expression related with poor prognosis. These findings are consistent with previous reports. Therefore, the reduced CD9 expression is generally related to more venous vessel invasion and metastasis as well as poor prognosis in most common types of cancer.

As mentioned above, many investigators have believed that CD9 is a suppressor of tumor development.

**POSIBILITY OF CD9-TARGETED THERAPY IN GASTRIC CANCER**

Anti-CD9 mAbs, ALB6 and PAINS-13 are ligand-mimic Abs, therefore, Ab ligation of CD9 with these antibodies enhances, but not inhibit, CD9 functions. We first introduce some interesting data concerning mechanisms of CD9 functions obtained by using these Abs. We previously reported that the treatment with anti-CD9 mAb (ALB6), which enhanced CD9 functions, inhibited cell growth in CD9-positive tumor cell lines (MKN-28, MKN-45, SW480, HT-29, CaCO2, MIA-PaCa-2, A459)[31]. In a gastric cancer line MKN-28, CD9 ligation induced apoptosis. ALB6 treatment activated JNK/SAPK and p38 MAPK as well as caspase-3[31]. Notably, ALB6 treatment selectively induced tyrosine phosphorylation of the p46 Shc isoform, and overexpression of its dominant-negative form completely cancelled the ALB6-induced activation of JNK/SAPK, p38 MAPK and caspase-3, leading to loss of apoptosis. Therefore, Ab ligation of CD9 induced apoptotic signals *via* restricted activation of the p46 Shc isoform. We also reported that CD9 ligation enhanced the internalization of EGFR[16]. ALB6 treatment induced a dotted or patch-like aggregation composed of CD9-EGFR and CD9-1 integrin on the surface of MKN-28 cells. Furthermore, expression of CD9 specifically attenuated EGFR signaling in CD9-overexpressing CHO cells *via* the downregulation of surface expression of EGFR[16]. Therefore, CD9 expression negatively regulates cell surface EGFR expression levels. Finally, we examined *in vivo* effects of ALB6 Ab to treat patients with gastric cancer. MKN-28 cells were inoculated subcutaneously into SCID mice. After a tumor was visualized, the MKN-28-bearing mice were injected with ALB6 or control Ab three times per week. In the ALB6 treatment group, tumor volume was significantly suppressed, and the apoptotic indexes were increased. Therefore, administration of mice bearing human gastric cancer cells with anti-CD9 Ab successfully inhibited tumor progression[52]. Similar to our results, it has been reported that anti-CD9 mAb PAINS 13 inhibited *in vivo* tumour growth of colon cancer cells[53]. The inihibition of cell proliferation in colon carcinoma cells caused by anti-CD9 mAbs PAINS‑13 was related to the enhanced integrin-dependent adhesion and the increased expression of membrane TNF-α.

Therefore, TNF-α partly mediates the anti-proliferative effects of CD9 in this case.

Overexpression of vascular endothelial growth factor-A (VEGF-A) is associated with tumor angiogenesis, nodal metastasis, and a poor prognosis in cancer patients[54,55]. A report that CD9 gene transduction could downregulate VEGF-A expression is now available[36]. In this situation, CD9 is also likely to negatively regulate tumor development.

Involvement of CD9 on integrin function, it seems to be complex and bi-directional on tumor development. The enhancement of integrin-mediated cell adhesion by CD9 not only inhibits metastasis and invasion of tumor cells but also contributes to cell adhesion-mediated drug resistance[56].

**PRESENT TREATMENTS FOR PATIENTS WITH GASTRIC CANCER**

Improving molecular characterization has translated into better survival in select patients with advanced gastric and esophageal cancer. Trastuzumab, an antibody targeting the anti-human epidermal growth factor receptor 2 (HER2) extracellular domain, induces antibody-dependent cellular cytotoxicity and inhibits the HER2 downstream signals. In the ToGA study, standard chemotherapy regimens (capecitabine plus cisplatin or fluorouracil plus cisplatin) combined with trastuzumab resulted in a longer survival time than standard regimens without trastuzumab in patients with HER2-positive gastric cancer[57,58]. In addition, ramucirumab, a monoclonal antibody targeting vascular endothelial growth factor receptor (VEGFR)-2, is the first biological treatment that showed survival benefits as a single-agent therapy for the second line chemotherapy (REGARD trial) in patients with advanced gastric cancer who progressed after first-line chemotherapy[59]. An early report of the phase III RAINBOW trial, testing ramucirumab in combination with paclitaxel for the second line therapy after platinum–fluoropyrimidine failure, also demonstrated an overall survival benefit of 9.6 mo *vs* 7.4 mo as compared with paclitaxel alone[60]. With recent success of ramucirumab, investigations with several other anti-angiogenic agents have begun. These include the VEGFR-2 inhibitor, apatinib, and the multi-targeted tyrosine kinase receptor inhibitors, axitinib and pazopanib[60]. In addition to the HER family and VEGFRs, the phosphatidylinositol-3-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) and the MET signaling pathways that are promising candidates, some molecular targeting agents are now in clinical investigation[61].

**FUTURE PROSPECTS**

A number of recent reports have suggested that tetraspanins targeting by Ab, soluble large-loop proteins or RNAi technology, adenoviral transduction methods could be therapeutically beneficial[62]. In the case of CD9, we and others have proposed that CD9 ligation is likely to be useful to treat malignancies. Ectopic expression of CD9 in small-cell lung carcinoma cells resulted in the inhibition of their proliferation[63], and adenoviral transduction of CD9 inhibited lymph node metastasis in an orthotopic lung cancer model[40]. With cDNA expression microarray experiments, CD9 was reported to be one of the genes up-regulated in gastric cancer[64]. Thus, CD9 expression in non-cancerous tissues is lower than that in gastric cancer tissues, indicating that adverse effects of anti-CD9 Ab-treatment on normal gastrointestinal tissues might be tolerable.

Tumor growth is dependent on angiogenesis, which forms new blood vessels[65]. Targeting tumor vessels provides several advantages over traditional anti-tumor approaches. CD9 enhancement contributes to tumour angiogenesis, presumably by affecting endothelial cell function although their contributions to angiogenesis have not been shown using *de novo* tumour models. It was previously reported that CD9 gene transduction could downregulate VEGF-A expression, which was essential for angiogenesis[36]. Therefore, enhancement of CD9 functions may also be worthy in particular circumstances.

With regard to tumor metastasis, CD9 is involved in cell adhesion *via* enhancing integrin functions. In addition, associations of CD9 with EWI-2[10,11,13,66,67], EWI-F[68,69], EPCAM[72], Claudin-1[10] or HB-EGF[23,24] could have very different effects on tumor cell invasion and metastasis. Indeed, the CD9 partners EWI-F[71] and EWI-2 can markedly affect cell migration[72], and EWI-2 influences the association of CD9 with membrane-type 1 matrix metalloproteinase (MT1‑MMP; also known as MMP14) and MMP2[73], which could alter proteolysis during invasion. Thus, CD9 acts on multiple steps of tumorigenesis, and because CD9 functions is dependent on its associating proteins, efficacy of the CD9-targeting therapy may be determined by expression of these associating molecules as well as CD9 itself.

**CONCLUSION**

Molecular mechanisms for CD9 functions have been understood through identification of CD9-associating proteins. Ab ligation of CD9 is a powerful tool to change CD9 functions, and we showed apoptotic signals after CD9 ligation in gastric cancer cells as well as successful treatment of gastric cancer-bearing mice with anti-CD9 Ab. CD9 influences intra-cellular signals, cell adhesion, and cell proliferation, and is involved in several events during development of gastric cancer. Taken together with evidence from clinical data, the manipulation of CD9 is likely to have the potential to improve clinical results of therapies for gastric cancer. When implementing CD9 target therapy to gastric cancer, we should come up with various ideas to enhance CD9 functions.

A new therapy to target HER2, VEGFR-2 is responsible for a significant increase in survival of patients with advanced gastric cancer. Unfortunately, advanced gastric cancer continues to have a poor prognosis. In the future, new strategy to target CD9 will hopefully be developed and implemented for gastric cancer treatment.

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**Figure 1 Structural features of CD9.** CD9 has four putative transmembrane domains, which provide the short N- and C-terminal cytoplasmic domains, a small intracellular loop, and two extracellular loops. C: Cysteine; G: Glycine.　

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**Figure 2 CD9 signalling.** CD9-EGFR and CD9-β1 integrin colocalize on the cell surface. CD9 enhances the internalization of EGFR and reduces EGF-EGFR-induced signals[11]. CD9 ligation induced apoptosis *via* the selective activation of JNK and p38 MAPK pathway as well as caspase-3 and the p46 Shc isoform[26]. CD9 modulates integrin-dependent cell motility, cell migration, adhesion strengthening and cell spreading[5,11]. EGFR: Epidermal growth factor receptor; p38 MAPK: p38 mitogen-activated-protein kinase; JNK: c-Jun NH2-terminal kinase; FAK: Focal adhesion kinase.

**Table 1 CD9 associated with partner proteins**

|  |  |  |
| --- | --- | --- |
| **Partner protein** | **Function** | **Ref.** |
| EWI-2 | Modulates integrin-dependent cell motility, morphology and/or spreading | [5-6,8,44,45,50] |
| EWI-F | Functions unknown | [5-6,8,46,47] |
| Integrin β1 | CD9 modulates integrin-dependent cell morphology, cell migration, signalling and adhesion strengthening | [5,11] |
| Other tetraspanins (*e.g.,* CD81, CD151) | Form TEMs | [7,8] |
| Claudin-1 | CD9 stabilizes expression of non-junctional Claudin-1 | [10] |
| EGFR | CD9 enhances the internalization of EGFR and reduces EGF-EGFR-induced signals | [11] |
| HB-EGF | CD9 upregulates both diphyteria toxin binding and mitogenic functions of HB-EGF | [23,24] |
| PKC isoforms | contribute to signaling and tumour-suppressor functions | [29] |
| Type II PI4K | contribute to signaling and tumour-suppressor functions | [30] |

EGFR: Epidermal growth factor receptor; HB-EGF: Heparin-binding epidermal-growth-factor-like growth factor; PKC: Protein kinase C; TEMS: Tetraspan-enriched microdomains.