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PRELIMINARY COMMUNICATION

Netrin-1 concentrations in patients with advanced gastric cancer and its relation with treatment

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Abstract

Context: Netrin-1 is found to be elevated and usable as a diagnostic biomarker in many human cancers.

Objectives: We evaluated serum Netrin-1 concentrations in patients with advanced gastric cancer compared with those in a healthy group.

Material and methods: Thirty patients with advanced gastric cancer and thirty healthy people were included in the study. Serum netrin-1 concentrations were measured by quantitative ELISA method in both groups.

Results: The mean serum Netrin-1 concentrations were found to be significantly higher in patients with gastric cancer than in healthy controls. The mean serum Netrin-1 concentrations were found to be significantly higher in patients with gastric cancer before the beginning of chemotherapy when compared after the completion of third cycle.

Discussion and conclusion: Our results indicated that netrin-1 concentrations elevated in advanced gastric cancer compared to a healthy control group and netrin-1 concentrations decreased with chemotherapy.

Keywords: Netrin-1, gastric cancer, treatment

Introduction

Netrin-1, a diffusible laminin-related protein, has been shown to play a major role in the control of neuronal navigation during the development of the nervous system (Arakawa 2004, Mehlen & Furne 2005, Fitamant et al. 2008). Recent studies have shown that netrin-1 is expressed outside the nervous system and contributes to the patterning of developing epithelial tissues by regulating diverse processes including adhesion, motility, proliferation, and differentiation of cells (Llambi et al. 2001, Ly et al. 2005, Wang et al. 2008, Rosenberger et al. 2009, Mehlen & Guenebeaud 2010, Mirakaj et al. 2010, Ramesh et al. 2011). Moreover, netrin-1 is known to regulate inflammation, angiogenesis, and apoptosis and therefore also takes place in the regulation of tumorigenesis by interacting with its main receptors, DCC (Deleted in Colorectal cell) and UNC5H (uncoordinated-5-homolog) (Arakawa 2004, Llambi et al. 2005, Ly et al. 2005, Mehlen & Furne 2005, Wilson et al. 2006, Nguyen & Cai 2006, Wang et al. 2008, Navankasattusas et al. 2008, Ramesh et al. 2011).

Increasing observations demonstrate that some receptors, in addition to their "positive" signaling when their ligand is present, transduce a "negative" signal that induces apoptosis in the absence of ligand. These receptors are named "dependence receptors." The most notable is their ability to trigger two opposite signaling pathways: in the presence of ligand, these receptors activate classic signaling pathways implicated in cell survival, migration and differentiation. In the absence of ligand, they do not stay inactive, rather they elicit an apoptotic signal (Goldschneider & Mehlen 2010). DCC

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and the UNC5H proteins are also dependence receptors which regulate apoptosis; positively in the absence of netrin-1 or negatively in the presence of netrin-1. (Arakawa 2004, Goldschneider & Mehlen 2010). The netrin-1-mediated anti-apoptotic signal that inhibits p53-induced apoptosis implies that NTN1 (which encodes netrin-1) could function as an oncogene (Arakawa 2004).

Alterations in the expression of netrin-1 occurs in various tumors. The expression of human NTN1 is markedly reduced or absent in ~50% of brain tumors and prostate cancer (Meyerhardt et al. 1999, Latil et al. 2003). Netrin-1 is found to be overexpressed in many cancer tissues (Bernet et al. 2007, Fitamant et al. 2008, Delloye-Bourgeois et al. 2009). Ramesh et al. showed that netrin-1 levels is significantly increased in many cancer types and secreted into circulation. They concluded that netrin-1 can be used as a biomarker of many human cancers and may be useful as a prognostic tool as well (Ramesh et al. 2011). Therefore, we investigated whether netrin-1 is increased in patients with gastric cancer, netrin-1 values change with treatment and netrin-1 is related with survival.

Materials and methods

Patient population

A total of 30 patients with histologically confirmed gastric cancer who were chemo-therapy-naive and followed-up at Dr. Lutfi Kirdar Kartal Education and Research Hospital, Department of Medical Oncology. All patients were staged as locally advanced or metastatic according to the AJCC/UICC TNM staging classification for gastric cancer (Greene & Fleming 2002). Patients were required to have an Eastern Cooperative Oncology Group (ECOG) performance status of ≤2, and laboratory requirements for the inclusion were having normal levels of bilirubin, hepatic enzymes, and renal functions. Hematologic requirements were a white blood cell count >3000 mm^3, absolute neutrophil count (ANC) >1000 mm^3, and platelets >100,000/mm^3. Moreover, 30 age- and sex-matched healthy people without any known malignancy who were selected between healthy relatives of patients were constituted as the control group. The Local Ethics Committee of our hospital approved the study, and the informed written consents were obtained from each patients and healthy controls.

Collection of samples

Five millimeters of peripheral blood samples were collected from 30 healthy people and 30 patients with advanced gastric cancer into the dry tubes, and samples were centrifuged at 1000*g* for 15 min to obtain serum within the half an hour after blood sampling. Patients' samples were collected prior to the chemotherapy and after the completion of third cycle. Netrin-1 levels were measured in both groups, and mean values were compared after all samples were analyzed simultaneously.

Measurement of netrin-1 concentrations by ELISA

A netrin-1 ELISA kit (Cusabio Biotech Co., Wuhan, China) were used to measure netrin-1 concentrations. Netrin-1 standard and plasma samples were incubated for 2 h at 37°C. 100 µL of biotin-antibody working solution was added and incubated for an additional 1 h. Each were aspirated and washed for a total of three times. Then 100 µL of horseradish peroxidase (HRP)–avidin was added and incubated for 1 h at 37°C. Again, each were aspirated and washed for five times. Ninety µL of tetramethylbenzidine substrate (TMB) was added and incubated for 30 min at 37°C. Then, 50 µL of Stop Solution, which was provided inside the kit, was added and the optical density (OD) was determined by using a microplate reader set to 450 nm. All measurements were averaged with duplicated readings. The concentration of netrin-1 in the samples was determined by comparing the OD of the samples to the standard curve, with a minimal limit of detection of 7.8 pg/mL. Netrin-1 concentration was expressed as picograms per milliliter.

Statistical analysis

Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) software. Descriptives of the parameters are quoted as median. The study parameters were normally distributed, therefore parametric tests were used. The mean concentrations of the samples were analyzed by Paired samples *t*-test if the variables are dependent and by Independent samples t-test if the variables are independent. Survival analysis and curves were established according to the Kaplan–Meier method and compared by the log-rank test. PFS was defined as the time from diagnosis to the last follow-up and the time until relapse as being the time since diagnosis to the first evidence of relapse. In addition, OS was described as the time from diagnosis to the date of the patient's death or last known contact. All *p* values were two-sided in tests, and *p* values less than or equal to 0.05 were considered to be statistically significant.

Results

Patient characteristics

Thirty patients with advanced gastric cancer and 30 healthy subjects as control group were analyzed. Twenty-two patients (73.3%) were men and 8 (26.7%) were women, with a median age of 58 years (range; 45–80 years). The majority of patients ($n = 28$, 93.3%) were metastatic disease, and the most frequent site of metastases (43.3%) was liver. In addition, the moderately differentiated tumors were the most common type of differentiation (63.3%). Histopathological subtypes of the patients were adenocarcinoma in 70.0% of patients, while there were only five signet-ring cell carcinoma and four mixed type. Twenty-three of the patients (76.7%) had a PS score of 0–1. Patient characteristics are listed in Table 1. In

Table 1. Patient characteristics.

Characteristics	*n*
Age	
Median (range)	58 (45–80)
Sex	
Men	22 (73.3%)
Women	8 (26.7%)
Performance score	
PS 0–1	23 (76.7%)
PS 2–4	7 (23.3%)
Tumor site	
Upper	19 (63.3%)
Middle	6 (20.0%)
Lower	5 (16.7%)
Surgery	
Present	3 (10.0%)
Absent	27 (90.0%)
Clinical stage	
Locally advanced	2 (6.70%)
Metastatic	28 (93.3%)
Histopathology	
Adenocarcinoma	21 (70.0%)
Signet-ring cell carcinoma	5 (16.7%)
Mixed type	4 (13.3%)
Tumor differentiation	
Well differentiated	2 (6.70%)
Moderately differentiated	19 (63.3%)
Poorly differentiated	8 (29.6%)
Site of metastases	
Liver	13 (43.3%)
Peritoneum	6 (20.0%)
Para aortic lymph nodes	4 (13.3%)
Mediastinum	4 (13.3%)
Lung	1 (3.30%)
Chemotherapy regimens	
DCF	22 (73.3%)
Capecitabine-Cisplatin	4 (13.3%)
DC	2 (6.70%)
Capecitabine-Carboplatin	1 (3.30%)
ECF	1 (3.30%)

the control group, the median age was 55 years (range: 43–77) and 20 healthy subjects were men (66.7%).

Netrin-1 concentrations

The mean netrin-1 concentrations were found to be significantly higher in patients with gastric cancer than in healthy controls (808.02 ± 514.23 vs. 507.13 ± 390.84, $p < 0.02$) (Figure 1). The mean netrin-1 concentrations were found to be significantly higher in patients with gastric cancer before the beginning of chemotherapy when compared after the completion of third cycle (808.02 ± 514.23 vs. 325.14 ± 441.98, $p < 0.001$) (Figure 2). There was no relationship between the decline in serum netrin-1 concentrations and clinopathological factors. The relationship between basal netrin-1 concentrations and gender, the presence of surgery, histopathological subtypes, stage, tumor differentiation, treatment response were not

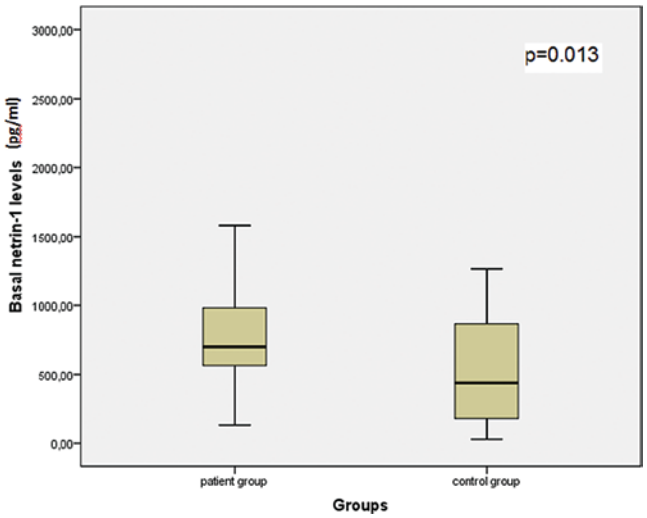

Figure 1. Mean concentrations of netrin-1 in patients compared with healthy control group.

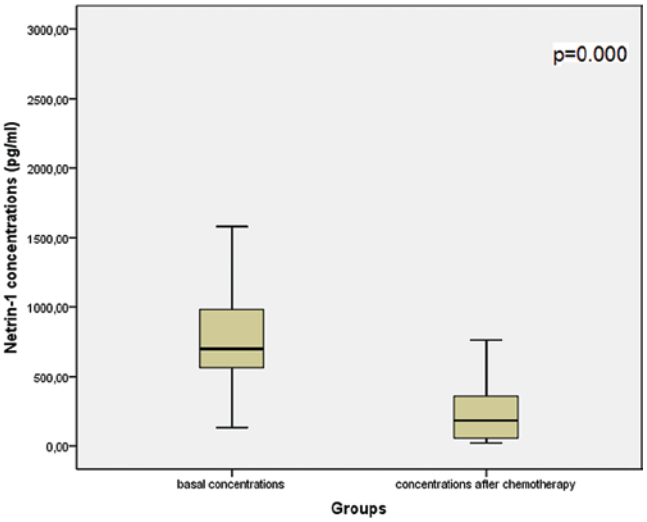

Figure 2. Mean netrin-1 concentrations in patients with advanced gastric cancer before the beginning and after the third cycle of the chemotherapy regimen.

significant ($p > 0.05$); however, we detected a significant correlation between age and basal netrin-1 concentration (Table 2). Thus, basal netrin-1 concentrations were significantly greater in cases under the age of 50 years (1166.0 ± 843.93 vs. 699.08 ± 318.03, $p < 0.04$).

Survival analysis

The median progression-free survival and overall survival of the patients were 4.46 months (95% CI = 3.83 –5.09) and 5.98 months (95% CI = 5.25–6.70), respectively. There were no significant correlations between clinicopathological factors, such as the presence of metastasis, histopathological type, clinical stage, tumor differentiation, tumor localization, patient's age, gender, and PFS ($p > 0.05$). Similarly, the relationship between clinicopathological factors and OS was not detected in the univariate analysis ($p > 0.05$). There was no relationship between the basal netrin-1 and decline in netrin-1 concentrations

Table 2. The relationship between basal netrin-1 concentrations and clinopathological characteristics of the patient group.

Variables	*n*	*p*
Age		0.033
<50 years	7 (23.3%)	
>50 years	23 (76.7%)	
Gender		0.22
Men	22 (73.3%)	
Women	8 (26.7%)	
Performance score		0.51
PS 0–1	23 (76.7%)	
PS 2–4	7 (23.3%)	
Tumor site		0.66
Upper	19 (63.3%)	
Middle	6 (20.0%)	
Lower	5 (16.7%)	
Surgery		0.58
Present	3 (10.0%)	
Absent	27 (90.0%)	
Clinical stage		0.70
Locally advanced	2 (6.70%)	
Metastatic	28 (93.3%)	
Histopathology		0.92
Adenocarcinoma	21 (70.0%)	
Signet-ring cell carcinoma	5 (16.7%)	
Mixed type	4 (13.3%)	
Tumor differentiation		0.71
Well differentiated	2 (6.70%)	
Moderately differentiated	19 (63.3%)	
Poorly differentiated	8 (29.6%)	

and the progression-free survival and overall survival ($p > 0.05$).

Discussion

Netrin-1 and its receptors are implicated in tumorigenesis via the regulation of tumor cell migration, tumor cell survival and tumor angiogenesis. The netrin 1 receptors, DCC and UNC5H are dependence receptors. They actively trigger apoptosis in the absence of netrin-1 (Mehlen et al. 2011). Such a trait confers on these receptors a tumor suppressor activity. It is a selective advantage for a tumor cell to lose this dependence receptor activity, as previously described with losses of DCC and UNC5H expression in human cancers. Earlier studies have documented that netrin-1 is overexpressed in breast (Fitamant et al. 2008), colorectal cancer (Paradisi et al. 2009), lung cancer (Delloye-Bourgeois et al. 2009), melanoma (Kaufmann et al. 2009), pancreatic cancer (Dumartin et al. 2010), and brain tumors (glioblastoma) (Mirakaj et al. 2010). However, whether overexpressed netrin-1 from tumors is secreted into the circulation and the levels of circulating netrin-1 were unknown. Ramesh et al. showed that netrin-1 is overexpressed in many tumors and secreted into the circulation. In their study, elevated levels of netrin-1 in plasma was seen in several tumors at all stages of tumor progression as compared to control patients. Their results suggested that plasma netrin-1 could be used as diagnostic biomarker of many human cancers (Ramesh et al. 2011).

Inhibition of apoptosis occurs by either a decrease in the levels of the receptors [e.g. DCC and UNC5H expression is lost in the majority of colorectal cancers] (Bernet et al. 2007, Fitamant et al. 2008, Paradisi et al. 2009, Mehlen & Guenebeaud 2010, Ramesh et al. 2011) or overexpression of netrin-1. Recent studies have shown that overexpression of netrin-1 confers a selective advantage for tumor cell survival in metastatic breast cancer (Fitamant et al. 2008). Hibi et al. found that aberrant methylation of the DCC and UNC5H gene was detected in primary gastric carcinomas (Hibi et al. 2009, Hibi et al. 2010). Chen et al. evaluated the relationship between netrin-1 protein, clinicopathologic features and prognosis in gastric cancer patients. They found that netrin-1 overexpression in tumor tissue was increased in patients with gastric cancer and might be related to the with tumorigenesis. However, netrin-1 expression was not significantly correlated with the prognosis of gastric cancer (Chen et al. 2011). In our study, we found that netrin-1 concentrations in blood was increased in patients with advanced gastric cancer. This is the first study that netrin-1 is found increased in circulation of patients with advanced gastric cancer. Although it can be thought that lysis after chemotherapy could cause false rise of netrin-1 concentrations after chemotherapy there was a statistically significant decline in netrin-1 concentrations after third cycles of chemotherapy when compared with basal netrin-1 concentrations. However, we found no relationship between netrin-1 concentrations, decline in netrin-1 concentrations after chemotherapy and survival.

Early age was proposed to be a high risk factor for patients with gastric cancer (NCCN 2011). In our study, we found that netrin-1 concentrations were higher in patients under the age of 50 years with advanced gastric cancer but there was no relationship between age and survival. This relationships should be further evaluated with other studies.

In this study, we analyzed patients with a known homogenous cancer type prospectively and there was no other obvious etiology for the elevated netrin-1 concentrations in blood. As discussed above, tumor lysis after chemotherapy which may result in a falsely rised netrin-1 concentrations were eliminated by collecting blood samples before operation and chemotherapy. Comparison of netrin-1 concentrations of patients with healthy people is another strength of this study.

Our study has also limitations. The major one was having limited number of patients. Although, variables were normally distributed, having 30 patients might have prevented to show statistical significances. Secondly, this study was done in patients after the diagnosis of gastric cancer. Thus, it should be validated in a larger population whether netrin-1 could determine the early diagnosis of gastric cancer. Another limitation

is the short follow-up period of the patients. Again, this might have prevented the results. Lastly, immunohistochemical localization of the netrin-1 and its receptor in the gastric tumor tissue were not evaluated in the present study. Therefore, we could not show whether there was a correlation between the rise of netrin-1 concentration in blood and the expression of netrin-1 and its receptors in tumor tissue.

Conclusion

Netrin-1 concentrations were elevated in advanced gastric cancer compared to a healthy control group and netrin-1 concentrations decreased with chemotherapy. But there were no relationship between netrin-1 concentration, decline in netrin-1 concentration and survival.

Declaration of interest

The authors have no conflicts of interest.

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