RESEARCH ARTICLE

Prognostic Significance of Expression of CD133 and Ki-67 in Gastric Cancer

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Abstract

CD133 is one of the most important stem cell markers in solid cancers and Ki-67 is a marker that reflects cell proliferation. The relationships between the expression of CD133 and Ki-67 and prognosis in gastric carcinoma are unknown and need exploring. We examined 50 gastric cancer patients retrospectively in the Radiation Oncology Department of the Faculty of Medicine, Gazi University. CD133 and Ki-67 expression was examined using immunohistochemical staining. The survival rate in patients with CD133 positive expression was significantly worse than that in the patients with negative expression (p=0.04). Expression of CD133 had a positive correlation with that of Ki-67 (r=0.350; p=0.014). Multivariate analysis revealed that the expression of CD133 was an independent prognostic factor in gastric cancer (p=0.02). Conclusion, expression of CD133 may be a useful prognostic marker in gastric cancer.

Keywords: CD133 - Ki-67 - gastric cancer - cancer stem cell - prognosis

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Introduction

Gastric cancer (GC) is one of the most common and most aggressive human cancers. According to the World Health Organization, GC is the fifth most common malignancy worldwide, and with an extremely poor prognosis, is the third leading cause of cancer death worldwide (Ferlay et al., 2013). Despite the fact that, GC is very common in Asian countries, it forms a major health problem in Turkey as well. GC is placed at the fifth order having 16% incidence in the cancer diseases seen among men according to 2009 Turkey data. Despite having radical resection and postoperative adjuvant therapy, most of GC patients will die of recurrence and metastasis, with 5-year overall survival ratio only 20% for resectable patients in Turkey. Another problem with GC is time of the diagnosis because generally by the time the patient is clinically diagnosed, the GC has often grown beyond the limits of curative resection. Also, according to Turkey data at the time of diagnosis 25% patients have distant metastasis, 48% patients have locoregional disease. Only 27% patients can be diagnosed with locally disease (Turkish Public Health Organization, 2009). Because of this fact, new early diagnostic tools and therapeutic techniques are needed.

Cancer stem cells, also known as tumor initiating cells, refer to a small side population of tumor cells with unlimited self-renewal activity and potentially promote the formation of tumor. CD133 is a 120 kDa glycoprotein with five transmembrane domains and is a cancer stem cell (CSC) marker (Reya et al., 2001). Although the biological function of CD133 is not well understood, it is considered as a regulating factor of membrane topology (Neuzil et al., 2007). Except of the hematopoietic stem cell, CD133 is also found in various solid tumors such as brain tumor (Zeppernick et al., 2008), colorectal cancer (Horst et al., 2008), ovarian cancer (Baba et al., 2009), pancreatic cancer (Li et al., 2007), prostate cancer (Collins et al., 2005), breast cancer (Zhao et al., 2011), lung cancer (Yao et al., 2014). In 2008 it was shown that a moderate to high percentage of GC tumor samples have CD133 expression with moderate to strong membranous and apical expression (Smith et al., 2008). But the prognostic importance of the CD133 in GC is still unclear. There is no effective treatment of highly advanced GC and recurrent GC. Therefore, identification of gastric CSCs and establishment of treatment will be highly important in future GC therapy.

Ki-67 is a well-recognized nuclear antigen-specific marker associated with cellular proliferation (Ross and Hall, 1995). Nowadays it is used not only an easy but also an effective method to show proliferation of many type of tumor. But results regarding Ki-67 have to be discussed. For instance, being high Ki-67 expression in invasive breast cancer as poor prognostic indicator was demonstrated by Yang et al. whereas, adversely being low Ki-67 expression in gastric cancer has poor prognosis was demonstrated by Arciero (Arciero, 2010; Yang et al., 2011). Therefore, in order Kİ-67 to be used as a routine

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marker, more study is needed.

In this study, demonstration of CD133 and Ki-67 expression, determination of their contribution to prognosis and establishment of correlation between Ki-67 and CD133 were aimed.

Materials and Methods

Patients

In this study, we examined 50 gastric cancer patients retrospectively who took chemoradiation therapy between June 2006 and April 2010 in Radiation Oncology Department of Faculty of Medicine, Gazi University. These patients' specimens were found in the same university, Pathology Department of Faculty of Medicine. The patient characteristics are shown in Table 1. The age of the patients ranged from 28-80 years, with an average of 56.2 years. There were 39 men and 11 women. All patients were advanced stage, level 2B-3C, according to 7th AJCC (American Joint Committee on Cancer). The surgery type was defined subtotal and total gastrectomy. The residual tumor was defined as R0; no residual tumor, R1; microscopic residual tumor, and R2; macroscopic residual tumor. Concerning lymph nodes, Lymph node involvement ratio was calculated by dividing patients' involved lymph node value by patients' lymph node value which was removed with operation. Patients are divided into three groups according to lymph node involvement ratio; 30% and lower, between 31% and 50% and 50% and higher.

The study was authorized in advance by the Cynical Research Ethic Committee of Faculty of Medicine, Gazi University in the meeting dated 23.03.2011.

Immunohistochemistry

All samples were fixed in 10% buffered formalin and embedded in paraffin. Sections were cut 4 μM thick from wax blocks, mounted on to poly-L-Lysine coated microscope slides. Deparaffization and antigen retroviral process were applied. Sections were incubated with blocking serum at room temperature for 20 minutes to protect nonspecific binding. CD133 (Milenyi Biotec Inc., CA, USA) diluted 1:20 were dropped in sections and kept in closed system for 1 hour at room temperature. Sections were incubated in 37°C water bath with Diaminobenzidine (DAP) as the chromogen for 10 minutes. Same process was repeated for Ki-67 (Milenyi Biotec Inc., CA, USA) and the samples were prepared. Fetal renal tissue was used as a positive control.

Table 1. Patient Information

Characteristics	Number of patients (n=50)
Age (mean), min-max years	56.2 (28-80)
Gender (male/female)	39/11
Localization of tumor (cardia, corpus, antrum)	11/10/29
Stage (IIB, IIIA,IIIB,IIIC)	7/12/12/19
Surgery (subtotal,total gastrectomi)	17/33
R(residual tumor-R0/R1 and R2)	33/17
Tumor size (mean), min-max cm	6.6 (1.6-15)
Removed lymph node number (mean), min-max	25 (4-87)
Involved lymph ratio ≤30%, 31%-50%, >50%	25/13/12

Statistical analysis

The overall survival rate and disease free survival rate were calculated using Kaplan-Meier analysis, and differences between the groups were compared using the log-rank test. For multivariate analysis, prognostic factors were analyzed using Cox's proportional hazard model. For comparison of categorical data, the chi-square test and Fisher's exact test were used. Two sided Spearman's correlation coefficient test was used for CD133 and Ki-67 correlation. All statistical analysis was made by SPSS 16.0 for Windows. A $p<0.05$ was considered statistically significant.

Results

Expression of CD133 and Ki-67 in gastric adenocarcinoma

When we looked at the ratio of CD133 expression, 3 samples had ratios more than 60%, 6 samples had ratios from 30% to 60% and 9 samples had ratios from 5% to 30%. These three groups were decided as CD133 positive expression (CD133+). 22 patients had not been painted, 9 patients had been painted lower than 5%. These two groups were decided as CD133 negative expression (CD133-). 2 Year overall survival (OS) in CD 133+ group was found as 24% while found as 57% in CD133- group. The difference between these two groups was statically significant (p=0.04). 2 Year disease free survival (DFS)

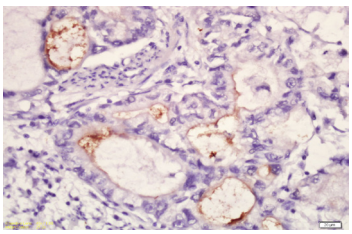

Figure 1. Expression of CD133 Protein in Gastric Adenocarcinoma. CD133 expressed positive in the membrane of cancer cells in adenocarcinoma (peroxidase, x200)

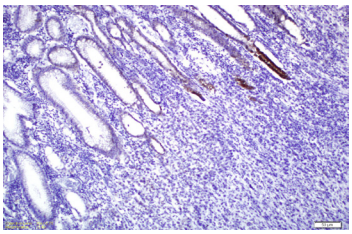

Figure 2. Expression of CD133 Protein in Signet Ring Cell Gastric Adenocarcinoma. CD133 expressed positive in the cytoplasm of cancer cells in signet ring cell adenocarcinoma which is known as bad prognosis type of gastric cancer (peroxidase, x100)

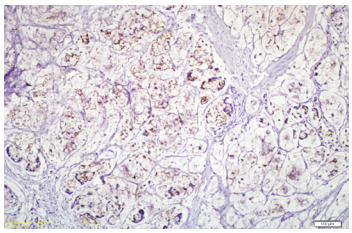

Figure 3. Expression of CD133 Protein in Mucinous Adenocarcinoma. CD133 expressed positive in the cytoplasm of cancer cells in mucinous adenocarcinoma (peroxidase, x200)

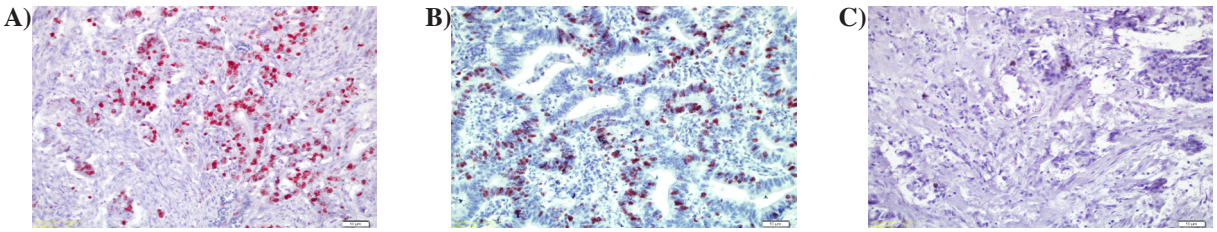

Figure 4. Ki-67 Expressed in the Nucleus of Cancer Cells. A) Adenocarcinoma showing 80% level Ki-67 positive (peroxidase, x100); **B)** Adenocarcinoma showing 50% level Ki-67 positive (peroxidase, x200); **C)** Adenocarcinoma showing 2% level Ki-67 positive (peroxidase, x100)

Table 2. Relationship between CD133, Ki-67 and Clinicopathological Features

		CD133		p	Ki-67		p
		+	-		+	-	
Age	≤60	12	20	0.88	24	8	0.12
	>60	6	11		9	8	
Tumor diameter	≤7cm	12	17	0.35	22	7	0.15
	>7cm	5	13		10	8	
Invasion depth	T3	1	4	0.64	5	0	0.16
	T4	17	27		28	16	
Lymph Node	N0	1	3	0.6	2	2	0.82
	N1	5	9		9	5	
	N2	3	9		9	3	
	N3	9	10		13	6	
Stage	II	1	6	0.24	5	2	1
	III	17	25		28	14	
ALI	Present	13	18	0.51	23	8	0.48
	Absent	0	2		1	1	
Grade	Low differential	13	15	0.22	22	6	0.62
	Middle differential	1	7		5	3	
	Good differential	1	2		2	1	

Table 3. Results of Multivariate Analysis

Paramater	95% Trust Ratio	p	Risk Ratio
Gender (male, female)	0.08-1.55	0.17	0.35
Tumor Place (antrum, others)	0.15-1.32	0.14	0.44
Tumor Diameter (≤7cm, >7 cm)	0.13-0.90	0.03	0.34
LN involvement ratio (≤30%, >30%)	0.05-0.45	<0.01	0.14
Resection Type (R0, R1-2)	0.17-1.26	0.13	0.46
CD133 (negative, positive)	0.09-0.80	0.02	0.27

in CD133+ group was found as 17% while found as 56% in CD133- group. However, on the contrary to OS, this difference was not statically significant (p=0.12). The location of CD133 was primarily in the membrane and cytoplasm of cancer cells. In Figures 1-3, some examples showing CD133 expression painting are demonstrated.

The ratio of Ki-67 was determinated as low and high painted groups. In high group, there were three subgroups: 6 samples were higher than 60%, 14 samples were between 30% and 60%, 13 samples were between 5% and 30%. In low group, 16 samples were painted 0-5%. There was no significant difference in both OS (p=0.94) and DFS (p=0.39) between low and high painted groups. Because of Ki-67 is specific at nucleus, its paint is in the cell nucleus. In 4a-4b-4c, there were three different painted values; 80%, 50%, 2%, respectively.

The association between CD133 and Ki-67 and also clinicopathological features

A mid-grade positive correlation between CD133 and Ki-67 was found (r=0.350; p=0.014). This means the higher CD133 expression ratio results in the higher Ki-67 ratio. There was no significant association between CD133 and clinicopathological features such as age, tumor size, invasive depth, lymph node, stage, angiolymphatic invasion (ALI) and grade. This result was the same as Ki-67 (Table 2). CD133 was an independent prognostic factor by multivariate analysis. Tumor size and ratio of the pathological lymph node were the other independent prognostic factors (Table 3).

Discussion

Cancer initiator cells or in other words cancer stem cells (CSC), which has unlimited self-regeneration and transformation feature to any cell, are known to increase tumor population as being proliferated (Jordan et al., 2006; O'Brien et al., 2008). With the start of CSC's examination, the question, asking whether tumor recurrence after accomplished treatment result from these cells, has come into minds. As a result of this, many animal studies have been made and these studies demonstrate that tumors having CSC have resistance to chemotherapy and radiotherapy. In spite of the fact that the exact reason to this resistance is unknown, in CSCs both transporter proteins forming multi medicine resistance on cell surface and genes preventing apoptosis are found to be relatively more which is thought to be responsible of this resistance (Setoguchi et al., 2004; Al Hajj, 2007; Tang et al., 2007; Tabarestani and Ghafouri-Fard, 2012). As in the case most solid tumors, results from researches made with CSC in head and neck cancer tumors demostrated that CSC positive cancer cells have relatively more migration, invasion and metastatic potential. In addition, it is determined that disease is more aggresive among these patient groups (Satpute et al., 2013). Nevertheless, for all cancer types related studies have been continuing on this subject.

It is possible to determine CSC due to some molecules on their surfaces. One of these molecules is cell surface molecule; CD133. Studies associated with CD133 have been coming from South Asia especially China in which gastric cancer is frequent. This study from Turkey; as a transition point from East to West; is important to show situation in different populations. In this study, CD133+ patient group is found to have lower relative OS in both single and multivariate analyses and in consequence of multivarate analyses CD133 is accepted as an factor (CD133+ OS 24%; CD133- OS 57%). DFS is found to be relatively higher (CD133+ DFS 17%; CD133- DFS 56%) but it has no meaning statistically. It was found to be compatible with many publications in the literature

that OS is low in CD133+ and CD133+ is evaluated as an independent prognostic factor (Ishigami et al., 2010; Zhao et al., 2010; Jiang et al., 2012; Lee et al., 2012). Lower OS ratio in CD133+ is a sign that CD133+ stem cells form resistance to treatment. For these patient groups, only cytotoxic medicine is not enough. Researches made by Yu et al. on mice showed that, CD133+ cells are less sensitive to chemotherapical drugs such as 5-FU than CD133- cells and CD133 gene expression inhibition led to significant increase in the sensitivity of CD133+ GC cells to 5-Fu (Yu et al., 2014). For this reason, nowadays targeted therapy methods are being researched. Expectations have been increased with cytotoxic medicine developed against CD133 gene or its product have been demonstrated as in vitro beneficial (Smith et al., 2008; Yu et al., 2014). It was detected with the studies made that CD133+ patients have relatively more tumor grade, nodal involvement, bigger tumor size, deeper invasion depth and advanced phase TNM stage (Ishigami et al., 2010; Zhao et al., 2010; Jiang et al., 2012). But in our study, no correlation can be found between CD133 and nodal involvement, tumor size, T stage, N stage, stage, ALI, histological grade. This is because all datas are from patients having chemoradiotherapy. All patients are stage IIB or over since chemoradiotherapy is applied in advanced stages. As distinct from our study, in other studies there were patients from all stages (stage 1-3). Therefore, it was found that once stage and lymphatic involvement increases cells with CD133+ also increases. The fact that all patients were having chemoradiotherapy and also advanced stage limited our study.

The location of CD133 was primarily in the glandular-luminal cell membrane surface expression (luminal expression, L-type) and cytoplasmic expression (C-type) of cancer cells. (Examples for both can be seen in Figure 1 and 2.) Originally, L-type CD133 was reported in colorectal cancer (Horst et al., 2008) and C-type CD133 was reported in pancreatic cancer (Madea et al., 2008). However, both in our study and Hashimato et al.'s study it was demonstrated that in gastric cancer both L-type and C-type expression exist at the same time. As distinct from our study, Hashimato et al. compared these two groups and showed that in gastric cancer C-type CD133 expression exists in more undifferentiated tumors and C-type is responsible from recurrence with treatment resistance (Hashimoto et al., 2014). In our study, due to the lack of luminal expression cases, evaluation was made without distinction between these two groups as Ishigami et al. (2010). Another important study regarding CD133 was a meta-analysis evaluating seven important studies made in the year of 2013. In this meta-analysis, patients from 742 asian population were examined. As the result of this analysis, 5-year OS was found to be 55% in patients with CD133-, while 21% in patients with CD133+. Yet, in this meta-analyis the decision couldn't be made whether CD133 is a stand alone prognostic factor or a combined prognostic factor with the other CSC's such as CD44. Therefore, the need was determined to have more studies with more patients and different stem cell markers. Besides, it was stated that, the study had a limitation since only asian population was examined and situation in western conditions was still unclear (Wen et al., 2013). In that respect, our study display importance as to show CD133 situation in differenet populations.

Nowadays one of the most important factors in cancer treatment having many studies in regards is Ki-67 index. Ki-67 is a well-defined particular to core antigen showing cell proliferation. Ki-67, expressed in growth and synthesis phases in cell cycle, doesn't get expressed in rest (G0) and early (G1) stages. Ki-67, informing about active cells in cell cycle, is in the present day shown to be increased in many tumors (Weidner et al., 1994; Scholzen and Gerdes, 2000). However, correlation between Ki-67 and survival is in fact not so clear. In gastric cancer, Arciero and Lee et al. found low Ki-67 index as poor prognostic whereas He et al. found high Ki-67 index as poor prognostic factor (Arciero 2010; Lee et al., 2010; He et al., 2013). In our study, there was no survival difference between patients with low and high Ki-67 indexes likewise Lazar et al. (2010) and Sanaat et al. (2013). Finally, correlation between Ki-67 and CD133 were shown in two studies and results were found different in these two studies. Yu et al. (2010) represented that Ki-67 is lower when CD133 is positive, namely Ki-67 and CD 133 have a negative correlation. On the contrary Zhao et al. (2010) represented that Ki-67 index ascends with CD 133 positive value and argued both as bad prognostic factors. In like manner Zhao et al. (2010) our studies are towards middle level positive correlation between Ki-67 and CD133. In our opinion, high values of Ki-67 have to be accepted as a bad prognostic factor since these values may cause rapid proliferation and tumor recurrence.

In conclusion, considering these data, CD133 is a prognostic marker for gastric cancer. By conducting studies in order to determine CD133 and similar markers and demonstrate their prognostic values, will yield to understand their impact mechanism and develop new therapy methods. With the help of advanced technology new molecular markers will become available in routine. Thus, by predicting prognosis of patients in advance, having more aggressive therapy and increase in survivals will be ensured.

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