RESEARCH ARTICLE

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Association of Two CD44 Polymorphisms with Clinical Outcomes of Gastric Cancer Patients

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Abstract

Objective: CD44 is an important cell adhesion molecule that plays a key role in growth, invasion, proliferation and metastasis of cancer cells. CD44 protein over-expression is associated with a poor prognosis of gastric cancer (GC) and previous studies have shown that CD44 gene polymorphisms could affect survival and recurrence. In this study, we tested the hypothesis that polymorphisms impacting on the CD44 signaling pathway may predict clinical outcomes in patients with GC. **Materials and Methods:** DNA was extracted from blood of 150 healthy individuals and formalin-fixed paraffin-embedded (FFPE) tumor tissue of 150 patients. The two polymorphisms rs187116 and rs7116432 were studied by RFLP-PCR and sequencing techniques. **Results:** There was a strong significant correlation between single nucleotide polymorphisms (SNPs) in the CD44 gene, tumor recurrence, and overall survival ($p < 0.0001$). The existence of a significant relationship between tumor recurrence and overall survival was proved in this study, with at least one allele G for the polymorphism rs187116 and at least one allele A for polymorphism rs7116432. **Conclusion:** These results provide evidence of a relationship between CD44 gene polymorphisms and clinical outcomes in our GC patients. This result could help identify individuals with GC who have a high risk of tumor recurrence.

Keywords: Gastric cancer- CD44 gene- single nucleotide polymorphism- RFLP-PCR

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Introduction

Approximately more than 930,000 cases with Gastric cancer (GC) were diagnosed throughout the world (Parkin et al., 2005). Overall survival in patients with GC is less than 14 months (Van Cutsem et al., 2009). The North West and North of Iran stand at a high risk regarding stomach cancer. The frequency of possible environmental risk factors and significant differences in the probability of GC in different regions of Iran, mark this country as an opportunity to investigate on GC etiology (Malekzadeh et al., 2009). In addition, among the patients who have enough time to undergo standard radio chemotherapy after stomach surgery, the relapse rate is worryingly high and equal to 40-60% (D'angelica et al., 2004). GC is a heterogeneous disease with two different histological subtypes, classified by Lauren as intestinal and diffuse, through which different molecular changes are explain (Lauren, 1965). However, people with GC were usually treated uniformly and regardless of histological and molecular subtypes. Depth of tumor invasion (Stage T), number of tumor infiltrated lymph nodes (Stage N) and pathologic differentiation represent the prognostic markers. Therefore, the creation of molecular markers is necessary in predicting the consequences of separate therapeutic strategies to maximize efficiency and minimize the side effects. CD44 is the main molecule for cellular connections, which plays an important role in a number of physiological processes including cell attachment, migration and growth regulation (Ponta et al., 2003). In humans, the CD44 antigen is encoded by the CD44 gene on chromosome 11, a complex gene formed of 20 exons. Half of the mentioned exons are expressed in most of the untransformed non

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activated cells as standard CD44 (CD44s), while the remaining 10 were incorporated in a number of CD44 family splice variants (CD44v) that contain inserts with variable sizes in the extracellular part of molecules. The CD44v were found on the surface of tumor cells and activate lymphocytes, demonstrating the distinct working importance of CD44s. CD44 signaling plays an important role in growth, invasion, proliferation and metastasis of cancer cell (Lin and Yang-Yen, 2001). Activation of CD44, which results from ligand binding, increases proliferation and resistance to apoptosis through phosphatidylinositol 3-kinase (PI3K)/Akt pathway (Bourguignon et al., 1997). Moreover, CD44 increases proliferating tumor cells in cooperation with membrane-bound receptor tyrosine kinases, containing C-src and C-erbB-2 (HER2/neu) (Bourguignon et al., 1999). Another important function of CD44 is to regulate tumor cell binding and motility. In active mode of CD44, cytoplasmic domain of CD44 can interact with actin cytoskeletons through ezrin, radixin and mosein (Family ERM) by increasing the membrane motility and migration of tumor cell (Gotley et al., 1996; Lesley et al., 2002). It is proven strongly that CD44 is involved in setting up and increasing activities, including the stimulation of cell growth, metastasis formation and the increase in gastric and colorectal cancer (Naor et al., 1997; Takaishi et al., 2009). In addition, CD44 positive GC cells are associated with the resistance to chemo- (5- Fluorouracil and etoposide) and radiotherapy which considers CD44 to be at least partly responsible for the resistance to treatment, resulting in early tumor recurrence (Shukla et al., 2009). According to expressed content, it is proven that genetic variants in the CD44 gene can affect the response to chemotherapy and human cancer incidence and survival (Takaishi et al., 2009; Vazquez et al., 2010). Significant progress in recent years has led the emergence of CD44 as a GC stem cell (CSC) marker (Takaishi et al., 2009). It has been reported that CD44 positive human GC cell lines are capable of self-renewal, longevity and multipotency (Takaishi et al., 2009). Considering the vast amount of evidence stating CD44 role in creating and enhancing a variety of tumorigenic processes and the complexity of CD44 gene and its splicing events, it is perfectly reasonable to consider this gene capable of harboring functional polymorphisms which may help further define GC sub-populations at high risk for early tumor recurrence. Therefore, this study intended to investigate the potential of CD44 gene polymorphisms as molecular prognostic markers for GC.

Materials and Methods

Patient samples and normal donors

A total of 300 subjects, including 150 GC patients as case group and 150 healthy individuals were investigated in this case-control study. Human GC tissues were collected from patients who underwent curative surgery at the Imam Reza and Omid Oncology Hospitals of the Mashhad University of Medical Sciences (MUMS), Mashhad, Iran from 1998 to 2010. Peripheral blood samples were obtained from 150 healthy subjects as normal controls; moreover, none of these individuals reported any type of cancers. Both the case and control subjects were Caucasian Iranians. The study was approved by the ethical committee of MUMS and written informed consent was obtained from all of the enrolled subjects before any procedures, as described before.

DNA purification and Genotyping

Tumoral formalin-fixed paraffin-embedded (FFPE) tissues collected from GC patients and also peripheral blood from healthy subjects were put into the EDTA tube. The genomic DNA was extracted from peripheral blood and FFPE tumoral tissue using the QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's protocol. Finally, the obtained DNA was stored at -20 °C for the following experiments. Polymerase chain reaction–restriction fragment length polymorphism PCR (PCR-RFLP) analysis was performed to determine the genotype for two selected SNPs of CD44 gene. Primer sequences, PCR products size and RFLP materials are summarized in Table 1.The PCR reaction solution had a volume of 20 µl and the thermal profile included an initial denaturation step of 95°C for 5 min followed by 35 cycles at 94 °C (30s), 61 °C (30s) and 72 °C (30s), and the final extension at 72 °C for 5 min. The CD44 PCR product was digested with the restriction enzyme NlaIII and MspI (Thermo Scientific, USA). The digested DNA products were analyzed by 3% agarose gel electrophoresis (Invitrogen, USA).

Single nucleotide polymorphisms (SNPs) and direct sequencing

We investigated two SNPs in CD44 gene sequence including rs187116 G>A and rs7116432 A>G according to literature review. The selected SNPs (rs187116 and rs7116432) are located in the exon 19 and intron 1 of CD44 gene, respectively. Our criteria in selecting a candidate gene polymorphism were the minor allele frequency (MAF) of the both selected SNPs 10% ≤ in Caucasians and the significant correlation with tumor recurrence and overall survival in papers. We performed direct sequencing in 4% of total samples to confirm the results of PCR-RFLP assay.

Statistical analysis

All the statistical analysis was carried out using SPSS 18.0 statistical software (SPSS, Chicago, IL, USA). Analyzing the age difference between patients and healthy subjects were done by Mann-Whitney test. The Kruskal-Wallis test, which compares three or more independent groups, was utilized for age differences and various states of SNPs. In order to compare the association between control and patient groups for genotype and allele frequencies, χ^2 test was applied. The Log Rank test was chosen to determine the factors that affect survival. All the P values< 0.05 were considered to be statistically significant.

Table1. PCR and RFLP Information for the SNPs of CD44

Polymorphism	Primer sequences (5′ → 3′)	PCR Product size (bp)	Restriction Enzyme	Location of polymorphisms
CD44	F: CATCGTCTTCTTGCTGTTAGGA	150	NlaIII	Exon 19
(rs7116432)	R: GGTCTTGGTTCAGGTAGGGAGA			þ779 A>G
CD44	F: AGGTGGTTGGAGATCACCTG	153	MspI	Intron 1
(rs187116)	R: CTTTCGCAAGAACCACTTCC			þ4883 G>A

Results

Study population

Three hundred subjects, including 150 GC patients and 150 control subjects were enrolled in this study, while the median follow up time was 4.8 months. We analyzed the demographic characteristics of the samples and observed that 75.3% (113 of 150) of the patients with GC and 71.34% (107 of 150) of healthy control were males, respectively. In addition, 37 (24.7%) of the GC patients and 43 (28.66%) of the healthy control were females. The mean age ± standard deviation (SD) of patient group and healthy control group were 63.22 ± 10.88 years (age range 40-82) and 58.3 ± 10.92 years (age range 34-78), respectively. There was no significant difference between the 2 subgroups according to age/gender distribution. The results of demographic features of the GC patients and healthy subjects are summarized in Table 2. There was no notable correlation between CD44 gene polymorphisms and clinical characteristic of GC patients including depth of tumor invasion, lymph node involvement, grade, and stage in GC patients. In order to investigate the SNP in an identical race in both case and control groups, all the subjects were Persian in our study. In addition, there was no significant correlation between polymorphisms of the CD44 gene and the type of tumor.

Genotype frequency of CD44 gene in two groups

All enrolled subjects were genotyped for two SNPs (rs187116, rs7116432) of CD44 gene. The frequencies of genotypes in the case and control group are presented in Table 3. When comparing GC patients with healthy blood donors, we found significant differences in genotypic frequencies between the patient and control groups for CD44 gene polymorphisms ($P < 0.0001$). Frequency of rs187116 AG and AA genotypes were 42.6% and 36.7% in the case group and 10.7% and 86% in healthy group, respectively. Moreover, in the case group, the frequency of GA and GG genotypes in rs7116432 polymorphism were respectively 42% and 36%, while in control group we had 6.7% and 92.7%. To check the results, six PCR product samples of SNPs were randomly selected for sequencing. As expected, the result of sequencing analysis confirmed the PCR-RFLP results (data not included).

Table 2. Demographic and Clinical Characteristic in Patients with Gastric Cancer and Healthy Subjects

Variable	Case group number 150 (%)	Control group number 150 (%)
Age (mean± SD)	63.22 ± 10.88 years	58.3 ± 10.92 years
Sex		
Male	113 (75.3%)	107 (71.34%)
Female	37 (24.7%)	43 (28.66%)
Grade		
P.D.	21 (14%)	
M.D.	80 (53.33%)	
W.D.	49 (32.66%)	
Lymph node metastasis (N)		
N0	19 (12.66%)	
N1/ N2/ N3	131 (87.33%)	
Stage		
I / II	53 (35.33%)	
III / IV	97 (64.66%)	
Depth of tumor invasion (T)		
≤ T2	30 (20%)	
> T2	120 (80%)	
Tumor type		
Intestinal	129 (86%)	
Diffuse	18 (12%)	

WD, Well differentiated; MD, Moderately differentiated; PD, Poorly differentiated, N0, No regional lymph node metastasis; N1, Metastasis in 1 to 2 regional lymph nodes; N2, Metastasis in 3 to 6 regional lymph nodes; N3, Metastasis in 7 or more regional lymph nodes

Table 3. Frequency of CD44 Genotypes (rs187116, rs7116432) between the Cases and Controls

SNP		Patients N (%)	Control N (%)	P value
	G/G	54 (36%)	139 (92.7%)	
rs7116432	G/A	63 (42%)	10 (6.7%)	< 0.0001
	A/A	33 (22%)	1 (0.6%)	
	A/A	55 (36.7%)	129 (86%)	
rs187116	A/G	64 (42.6%)	16 (10.7%)	< 0.0001
	G/G	31 (20.7%)	5 (3.3%)	

Table 4. CD44 Polymorphisms and TTR and OS in Patients with Gastric Cancer

SNP		Median time to tumor recurrence (95% CI)	P value*	Median overall survival (95% CI)	P value *
rs7116432	G/G	6.1 (5.38, 6.82)		5.4 (4.75, 6.05)	
	G/A	4.5 (4.39, 4.61)	< 0.0001	2.7 (2.48, 2.92)	< 0.0001
	A/A	4.3 (4.16, 4.44)		2.5 (2.22, 2.78)	
rs187116	A/A	6 (5.95, 6.05)		4.6 (3.63, 5.57)	
	A/G	4.3(4.2, 4.4)	< 0.0001	2.5 (2.33, 2.67)	< 0.0001
	G/G	4.3 (3.99, 4.61)		2.3 (1.97, 2.63)	

*, Based on the log-rank test

CD44 genotypes and clinical outcome in GC

The associations of CD44 polymorphisms with time to tumor recurrence (TTR) and overall survival (OS) in GC patients are presented in Table 4, while significant association was observed between rs187116 and rs7116432 gene polymorphisms. Patients with CD44 rs187116 AG and AA genotypes had a median TTR of 4.3 and 6 months (95% CI, 4.2- 4.4 and 5.95- 6.05 months) respectively, in comparison with 4.3 months (95% CI, 3.99- 4.61 months) in homozygous patients for GG genotype (p< 0.0001, log-rank test). In addition, patients with CD44 rs187116 AG and AA genotype reached borderline significance with median OS of 2.5 and 4.6 months, respectively (95% CI, 2.33- 2.67 and 3.63- 5.57 months) compared to 2.3 months (95% CI, 1.97- 2.63 months) regarding homozygous patients for GG genotype (p< 0.0001, log-rank test). CD44 rs7116432 GA and GG genotypes had median TTR of 4.5 and 6.1 months, respectively (95% CI, 4.39- 4.61 and 5.38- 6.82 months) compared with 4.3 months (95% CI, 4.16- 4.44 months) for AA genotype (p< 0.0001, log-rank test). Moreover, a median OS of 2.7 and 5.4 months (95% CI, 2.48- 2.92 and 4.75- 6.05 months) versus 2.5 months (95% CI, 2.22- 2.78 months; p< 0.0001, log-rank test), respectively (Table 4).

Multivariable analysis of CD44 rs187116 and CD44 rs7116432

Multivariable analysis of CD44 gene polymorphisms (rs187116 and rs7116432) in GC patients is demonstrated in Table 5. CD44 rs187116 AG and AA genotypes remained significantly associated with TTR (HR: 2.933, 95% CI, 1.85- 4.651; adjusted p = 0.001 and HR: 3.138, 95% CI, 1.848-5.33; adjusted p = 0.000), respectively. In addition, CD44 rs187116 AG and AA genotypes continued to be significantly associated with OS (HR: 2.671, 95% CI, 1.695- 4.21; adjusted p = 0.000 and HR: 2.464, 95% CI, 1.45- 4.188; adjusted p= 0.000), respectively. CD44 rs7116432 GA and GG genotypes remained notably associated with TTR (HR: 2.655, 95% CI, 1.638- 4.306; adjusted p = 0.000 and HR: 3.027, 95% CI, 1.777- 5.156; adjusted p = 0.000), respectively. In addition, CD44 rs187116 AG and AA genotypes remained associated with OS (HR: 2.63, 95% CI, 1.65- 4.191; adjusted p = 0.000 and HR: 2.156, 95% CI, 2.156- 6.26; adjusted p = 0.000), respectively. The significant associations between CD44 polymorphisms and type of tumor were observed, as type of tumor was significantly associated with TTR (HR: 2.209, 95% CI, 1.308- 3.731; adjusted p = 0.003).

Table 5. Multivariate Analysis of CD44 Polymorphisms and TTR and OS in Patients with Gastric Cancer

SNP		Time to tumor recurrence Hazard Ratio† (95% CI)	P value†	Overall Survival Hazard Ratio† (95% CI)	P value†
rs7116432	G/G	3.027 (1.777, 5.156)	0.000	2.156 (2.156, 6.26)	.000
	G/A	2.655 (1.638, 4.306)	0.000	2.63 (1.65, 4.191)	.000
	A/A*	1 (Reference)		1 (Reference)	
rs187116	A/A	3.138 (1.848, 5.33)	0.000	2.464 (1.45, 4.188)	.000
	A/G	2.933 (1.85, 4.651)	0.001	2.671 (1.695, 4.21)	.000
	G/G*	1 (Reference)		1 (Reference)	
Type of tumor	Diffuse	2.209 (1.308, 3.731)	0.003		
	Intestinal	1(Reference)			

*, Normal genotype is assumed as the reference; †, Wald test in Cox Proportional hazards model

Discussion

CD44 and its activator ligands, hyaluronan and osteopontin, interact with each other in a signaling network to launch with various tumorigenic processes involving several growth regulation, survival, differentiation and motility. In addition, many documents that were obtained in recent years strongly confirm the fact of CD44 as being a gastric CSC marker. This study indicates that CD44 polymorphisms, alone or combined, have a significant relationship with TTR and OS in patients with gastric cancer who are treated with only surgery or a combination of surgery and adjuvant (radio)-chemotherapy (Takaishi et al., 2009). These statistical associations were maintained after the adjustment regarding other potential predictors of patient outcome, being independent from the cancer stage and lymph node involvement in stomach cancer. Previous studies have shown that the over-expression of protein CD44 is associated with the weak recognition of colorectal cancer and gastric cancer (Takaishi et al., 2009). However, CD44 is created by a single gene; various splice variants are created by adjustments after transcription and alternative splicing (Ghaffarzadehgan et al., 2008). Exceptional attention was drawn when it was expressed that a particular CD44V has the potential to facilitate the creation of a metastatic phenotype in a variety of tumors including GC (Ghaffarzadehgan et al., 2008; Huh et al., 2009). It is not clear whether the particular modification directly impacts the binding affinity of CD44, as well as whether certain splice forms can facilitate oligomerization by increasing the CD44 affinity for ligands, Hyaluronan, and Osteopontin (Huh et al., 2009). The new findings prove that intron changes may affect the splicing regulatory elements that cause the aberrant splicing (Barbour et al., 2003). In addition, Narla et al has presented evidence in accordance with the relationship between relatively common intronic polymorphism and KLF6 that is a Kruppel-like zinc finger transcription factor, an alternative splicing in prostate cancer (Narla et al., 2005). Recently, Vazquez et al., (2010) prevailed that C/C genotype of intronic CD44 rs187115 was significantly associated with decreased cellular response to cytotoxic chemotherapeutics such as doxorubicin, carboplatin, and Anti-metabolite: DNA and RNA / DNA in vitro that demonstrates the functionally significant role of this SNP in tumor cells. Additionally, the functional significance of this polymorphism was confirmed in the analysis of 129 sarcoma soft-tissue patients, in which the homozygous patients for the Allele –C, in comparison with those had the genotype T/T or C/T, were more likely exposed to 2.1 times risk of death from tumor (p=0.041) (Vazquez et al., 2010). Amazingly, in our study, patients with GC that had G/G genotype of CD44 rs187116 were more exposed to death risk in comparison to patients with Genotype A/A. Moreover, in the current study, two CD44 intron-genetic variations (rs187116 and rs7116423) were reported that had significant association with OS or TTR in a single marker analysis regarding patients with Gastric cancer. Although the detailed biological and functional significance of these polymorphisms are still unknown, yet it is reasonable that CD44 intronic polymorphisms may have a direct impact on the regulation of gene splicing events. Next expression of alternate transcripts and CD44 receptor variants has been reported effective in launching and increasing tumorigenic processes. Based on our knowledge, there are no unpublished reports that might have described the functional role of CD44 intron polymorphisms, tested in our study. Therefore, molecular mechanisms, by which SNPs enter their biological effects, should undergo further laboratory investigations. However, these data highly confirm important clinical and functional CD44 role and genetic variants, as well as they may contain clinical-conversion relevance. Early clinical trials using anti- antibodies against CD44, which prevent ligand binding, indicate the somewhat promising idea of using these antibodies to prevent tumor growth and metastasis (Mstzku et al., 1991; Wielenga et al., 1993; Pagani and Baralle, 2004; Klingbeil et al., 2009). Although it has been found in some researches that in parts of the body with high CD44 level such as skin, various problems are produced due to different toxic substances (Tijink et al., 2006; Rupp et al., 2007; Zhuo et al., 2012; Qiu et al., 2014). Therefore, the challenge is to improve the antibodies associated with targeting them to CD44v, which the questioned cancer is expressed uniquely. Further clinical trials should be done in order to consider genetic variations available in gene CD44, while we tested this method in an effort to increase appropriate therapeutic strategies.

In conclusion, the results of this study can provide the first substantial evidence in relation to this issue that CD44 polymorphisms, alone or in combination can anticipate the early recurrence of patients' gastric cancer tumor. The latter point is possible to help in selecting patients who are at high risk for tumor recurrence, thus these people can benefit from the specific therapeutic strategies. To affirm and confirm the findings of our preliminary hypothesis, a clinical trial is needed in which biomarkers are used.

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The authors declare that they have no conflict of interest.

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