ONCOLOGY REPORTS 18: 1583-1587, 2007

SPANDIDOS PUBLICATIONS

Analysis of CXCL12 3'UTR G>A polymorphism in colorectal cancer

M. HIDALGO-PASCUAL[1], J.J. GALAN[2], M. CHAVES-CONDE[3], J.A. RAMÍREZ-ARMENGOL[4], C. MORENO[2], E. CALVO[3], P. PELAEZ[1], C. CRESPO[5], A. RUIZ[2] and J.L. ROYO[2]

[1]Servicio de Cirugía General-B, Hospital Universitario 12 de Octubre, 28041 Madrid; [2]Departamento de Genómica Estructural, Neocodex SL, Avda Charles Darwin 6 Acc A, Isla de la Cartuja, 41092 Sevilla; [3]Servicio de Oncología Médica, Hospital Universitario Virgen del Rocío, Avda Manuel Siurot, 41013 Sevilla; [4]Servicio Central de Endoscopia, Hospital Clínico San Carlos, 28040 Madrid; [5]Hospital Ramón y Cajal, Ctra Colmenar Viejo Km 9, 28034 Madrid, Spain

Received May 8, 2007; Accepted June 27, 2007

Abstract. Colorectal cancer is one of the most prevalent cancers in developed countries. However, the genetic factors influencing its appearance remain far from being fully characterized. Recently, a G>A functional transition mapping the 3' untranslated region of the CXCL12 gene (rs1801157) has been found to be under-represented among rectal cancer patients when compared to colon cancer patients from a Swedish series. Here we present the results from an independent analysis of CXCL12 rs1801157 in a larger CRC series of Spanish origin in order to analyse the robustness of this association within a different European population. No significant difference was observed between controls and colon or rectal cancer patients. We were also unable to find a correlation between rs1801157 and different prognostic markers such as metastasis development or disease-free survival time. The epidemiologic data involving CXCL12 rs1801157 in colorectal cancer risk are discussed.

Introduction

Colorectal cancer (CRC) is the second most common type of cancer in developed countries. Therefore, great investment is being made to gain new insight on how genetic and environmental factors contribute to its development and evolution (1). The role of chemokines has been extensively analysed both in cancer risk and tumor progression (2-6). Among different cytokines, CXCR4 and its ligand CXCL12 (previously designated SDF-1) have been recently subjected to a closer examination. The CXCL12-CXCR4 pathway has been traditionally associated to hematopoietic cells homing (7). However, the CXCL12-CXCR4 axis has been also found to be a survival or growth factor in a variety of normal and malignant cell types including germ, leukemia B and breast cancer cells (7-9). It has also been shown that under low oxygen levels such us those found inside the tumors, both CXCL12 and CXCR4 are significantly up-regulated. This feature has been correlated with a poor survival in different cancer series (10-13). In addition, CXCL12-CXCR4 crosstalk has been found to be crucial for metastasis appearance in breast cancer and colorectal models by inducing chemotactic and invasive responses (14,15). The expression of the CXCR4 gene in intestinal epithelial cells has been previously reported. Preliminary experiments in animal models showed a role of CXCL12-CXCR4 in CRC metastasis, particularly by triggering the outgrowth of micrometastases (16). This model would be eventually applicable to different solid tumor types, since high CXCR4 expression has been also observed in breast and prostate cancer among others (10,14,17,18).

One of the first results involving the CXCL12 pathway emerged from experiments performed in 1998 (19). It was known that CXCR4 acts as a coreceptor by the viral strains that emerge during late-stage HIV-1 infection. In a genetic association analysis comprising 2,857 patients from five different cohort studies, Winkler *et al* found that people homozygous for rs1801157 allele-A showed a significant delay in the onset of the disease (19). This effect was twice as strong as the dominant genetic restriction of AIDS conferred by CCR5 and CCR2 in these populations, and was independent and complementary to these mutations in delaying the onset of the disease.

Since its discovery, extensive literature has been published ascribing different effects associated to CXCL12 rs1801157 to different phenotypes where chemokines may be exerting a significant role (20-23). To date, different experiments have been assayed to determine the molecular basis of the effects attributed to rs1801157. Some data suggest that the A-variant may be affecting mRNA stability depending on the cellular background, which would result in higher protein

Correspondence to: Dr Jose Luis Royo, Departamento de Genómica Estructural, Neocodex SL, Avda Charles Darwin 6 Acc A, Isla de la Cartuja, 41092 Sevilla, Spain
E-mail: jlroyo@neocodex.es

Key words: colorectal cancer, SDF1-3'A, rs1801157, CXCL12, 3'UTR

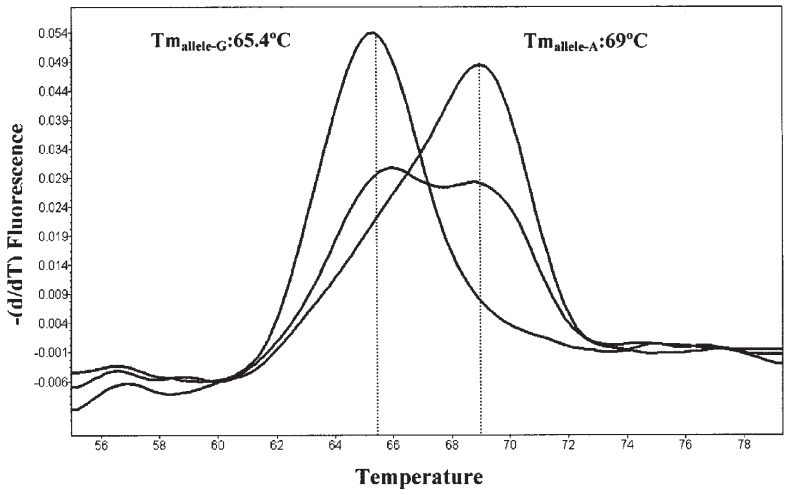

Figure 1. FRET analysis of CXCL12 rs1801157 alleles.

levels (Rueda P, *et al*, 13th International Symposium on HIV and Emerging Infectious Diseases, Toulon, France, 2004). However, no statistical correlation has been found between circulating levels of CXCL12 and the rs1801157 genotype (24).

In a recent report, Dimberg *et al* analysed the effect of CXCL12 rs1801157 in colorectal cancer risk (24). Using PCR-RFLP analysis, they genotyped a series of 151 CRC cases and 141 healthy unrelated controls from the general Swedish population. A preliminary analysis found no statistical difference between cases and controls. However, when patients were stratified according to the tumor location (colon vs rectum), they found a strong under-representation of the rs1801157 allele-A among rectal cancer patients when compared to the general population. To our knowledge, this was the first evidence linking CXCR12 alleles to CRC susceptibility, suggesting that the CXCL12-CXCR4 crosstalk could be somehow conditioning the primary tumor development and the metastasis generation processes. In this study, we show the results of an independent analysis of CXCL12 rs1801157 in a CRC series and a meta-analysis taking into account both colon and rectal cancer. To this end, we analysed the CXCL12 rs1801157 genotype distribution among 373 CRC patients and 558 controls from the Spanish population.

Patients and methods

Patients and controls. A total of 930 individuals were included in this study. Patients diagnosed between the ages of 35 and 87 years old were consecutively recruited between 2003 and 2005 from four different centres: Hospital Universitario San Carlos (Madrid), Hospital 12 de Octubre (Madrid), Hospital Virgen del Rocio (Sevilla) and Hospital Ramón y Cajal (Madrid). All patients and controls were mediterranean caucasians from central and south Spain. All subjects supplied written informed consent, together with a blood sample and completed a questionnaire regarding individual, environmental and familial variables. An institutional review board from the referral centres approved the protocol used during this study. DNA extraction was performed automatically according to standard procedures using the Magnapure LC DNA isolation system (Roche-Diagnostics, Germany).

CXCL12 rs1801157 genotyping. Genotypes were obtained using fluorescence resonance energy transfer (FRET) and LightCycler™ technology (Roche-Diagnostics, Germany), as described by Royo *et al* (25) with modifications. Briefly, polymerase chain reactions were performed at a final volume of 15 μl containing 10 ng of genomic DNA, 1 mM $MgCl_2$, 10 pmol of each primer (SDF1-F: 5'-TGGGTTTTGTATTCT CTGAGCT-3', and SDF1-R: 5'-AGCTTTGGTCCTGAGAG TCC-3'), 3 pmol of each genotyping probe (SDF1-sensor: Cy5-GAGCCAGGTCTGCCTCTTCTG-Ph, SDF1-Anchor 5'-GCTCACCCCCTTCTCCATCCACAT-Fluorescein) and 3 μl master mix of LightCycler-480 Genotyping Master (5X). PCR reactions were performed in MJ Research thermocyclers (MA, USA) with the following conditions: 95 min for 5 min followed by 35 cycles of amplification (95˚C for 30 sec, 63˚C for 30 sec, 72˚C for 30 sec) and finally a 3 min extension step. Then, plates were subjected to a melting curve in the LC-480 real-time PCR system while recording Cy5 fluorescence (95˚C for 30 sec and 55-80˚C curve with a slope of 2 measurements per ˚C). Raw data from FRET analysis are exemplified in Fig. 1. The call rate obtained was 94% for both cases and controls.

Statistical analysis. For statistical power studies and meta-analysis we used Episheet 2002 (26). For comparison of genotype distribution, test for deviation from Hardy-Weinberg equilibrium and two-point association studies, we employed tests adapted from Sasieni (27). These calculations were performed using the online resource at the Institute for Human Genetics, Munich, Germany (http://ihg.gsf.de) and Statcalc (EpiInfo 5.1, Center for Disease Control, Atlanta, GA). For Kaplan-Meier analysis, SPSS 14.0 was used.

Results

CXCL12 rs1801157 and CRC risk. Genotype distributions of rs1801157 in both series are shown in Table I. Allele frequency

Comparison of CXCL12 rs1801157 genotype distribution between the two independent series.

Study	Genotype	Controls	Cases		Colon cancer		Rectal cancer	
Dimberg *et al* (24)	GG	81	84	1.00 (Reference)	35	1.00 (Reference)	49	1.00 (Reference)
	GA	56	62	1.07 (0.66-1.71)	37	1.53 (0.81-2.72)	25	0.74 (0.41-1.33)
	AA	4	5	1.21 (0.31-4.65)	4	2.31 (0.55-9.78)	1	0.41 (0.05-3.80)
				0.731[a]		0.09[a]		0.22[a]
	MAF	0.23	0.24		0.30		0.18	
Present study	GG	319	212	1.00 (Reference)	126	1.00 (Reference)	77	1.00 (Reference)
	GA	172	128	1.20 (0.84-1.49)	68	1.00 (0.71-1.42)	50	1.20 (0.81-1.80)
	AA	25	9	0.52 (0.25-1.18)	5	0.51 (0.19-1.35)	3	0.50 (0.15-1.70)
				0.76[a]		0.42[a]		0.99[a]
	MAF	0.22	0.21		0.20		0.22	

[a]p_{trend} according to Armitage's trend test.

Table II. Comparison of CXCL12 rs1801157 genotype distribution according to gender.

Genotype	Male			Female		
	Controls	Cases		Controls	Cases	
GG	151	100	1.00 (Reference)	159	102	1.00 (Reference)
GA	68	71	1.58 (1.04-2.39)	98	47	0.75 (0.49-1.15)
AA	12	7	0.88 (0.34-2.31)	13	1	0.12 (0.02-0.93)
			0.17[a]			0.02[a]
MAF	0.20	0.24		0.23	0.24	

[a]p_{trend} according to Armitage's trend test.

of CXCL12 rs1801157 was almost identical between controls (allele-A: 0.22) and those reported by Dimberg *et al* for the Swedish population (allele-A: 0.23) (24), and indistinguishable from other general population studies previously described (25). Genotype distributions were concordant with the Hardy-Weinberg equilibrium law for controls but deviated among cases due to a slight defect of homozygotes for allele-A (p=0.67 and p=0.04 respectively, Pearson test), suggesting a role of CXCL12 rs1801157 in CRC risk. However, when case-control analysis was performed, no statistical difference was observed (Table I). Then, patients were stratified according to tumor location (colon vs rectum). When genotype distribution was compared, no statistical difference was observed between both subgroups neither in allele frequency nor genotype distribution (p>0.55). Thus, when studies were performed comparing genotype distribution between controls and colon or rectal cancer patients, no statistical difference was observed (Table I).

Genotype and allelic distributions in our CRC series did not differ when stratified according to Dukes stage or age of onset, as previously described (24). They also did not correlate with histological grade or tumor size (data not shown). On the other hand, we found an association of rs1801157 to CRC among females (Table II). We examined the correlation between the prevalence of colon or rectal cancer according to gender. In our series, neither sex was especially over-represented among colon cancer patients. However, females (47%) accounted for 40% of the rectal cancer patients whilst males (53%) accounted for 60% of them. When two-point cross-tabulation analysis was performed, we observed that this difference in the gender distribution was not statistically significant, but a trend towards association was observed (χ^2=3.68, p=0.05).

Next, we performed a statistical meta-analysis of both studies using a fixed effects model. All p-values for homogeneity tests ranged between p=0.09 and p=0.88. Thus, we performed a pooled meta-analysis taking into account both series, failing to correlate rs1801157 genotype distribution with neither colon nor rectal cancer risk (Fig. 2). However, we observed that among those patients with rectal cancer, both studies exhibited similar effect size trends.

CXCL12 rs1801157 in cancer progression. Given the well-characterized role of CXC12-CXCR4 axis in tumor metastasis development we postulated that rs1801157 could be associated

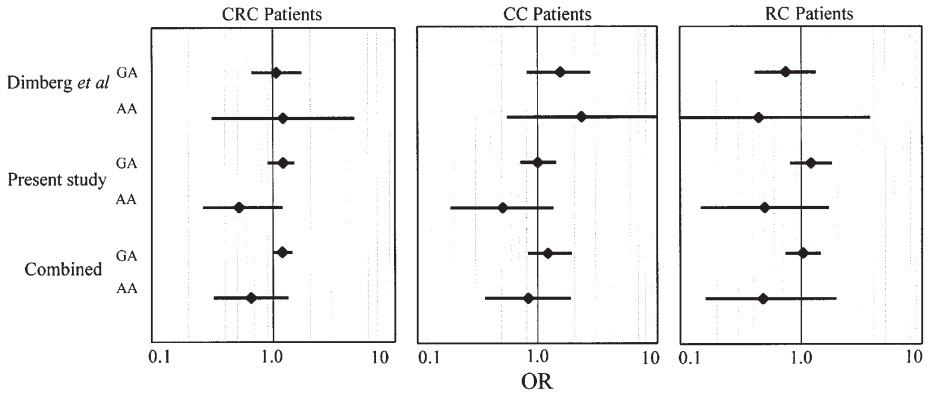

Figure 2. Graphical representation of the meta-analysis of CXCL12 rs1801157 in colorectal (CRC), colon (CC) and rectal (RC) cancer, comparing the present study with that of Dimberg *et al* (24).

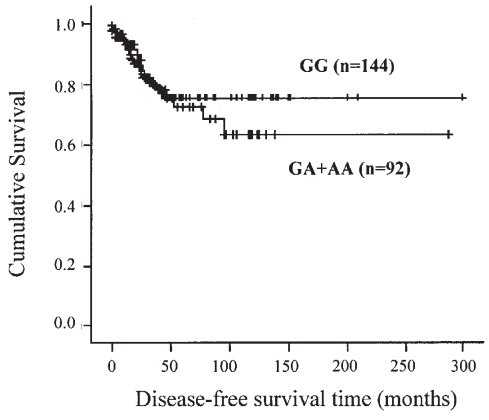

Figure 3. Kaplan-Meier analysis showing the role of CXCR12 rs1801157 in DFS time.

to a differential prognosis. Thus, we decided to study the putative involvement of rs1801157 in tumor aggressiveness. Of our patients, 14% presented metastasis at the moment of diagnosis and among those who did not, 12% developed metastasis in the following 5 years. Given previous results linking CXCL12 expression to metastasis generation, we analysed whether rs1801157 was associated to metastatic CRC. When genotype distribution was analysed within these groups, no difference was observed (data not shown). Finally, we performed a disease-free survival (DFS) time analysis in order to determine a putative correlation between the disease progression and rs1801157 using Kaplan-Meier analysis. The DFS mean times among those GA and AA patients was slightly lower in respect to the one exhibited by GG patients (Fig. 3). However, this difference was not statistically significant (Mantel-Cox log-rank test, p=0.63).

Discussion

Molecular data reported by several groups give no doubt about the oncogenic properties of the CXCL12-CXCR4 pathway. This has been demonstrated in different studies using both *in vitro* and *in vivo* experiments (14,16). In addition, the CXCR12-CXCR4 axis is a good candidate to be subjected to chemical intervention, which would eventually generate a new therapeutic approach for different tumors (28,29). For this reason, we find of great interest the elucidation of the potential role of CXCL12 in tumor development and progression. Previous data reported a significant difference in genotype distribution among colon cancer patients when compared to rectal cancer patients (χ^2: 5.6, p=0.02). In addition, a trend was observed when comparing controls to colon cancer patients (p_{trend}=0.09) and rectal cancer patients (p_{trend}=0.22). Thus, it seemed that the sample sizes compromised the robustness of the associations, although a clear trend was observed. For this reason, we performed a parallel analysis doubling the sample size, which according to our power calculations would be able to detect an odds ratio ranging the magnitude reported by Dimberg *et al* (24).

Here we have shown the results from an independent analysis of CXCL12 rs1801157 on CRC aetiology and the first data reporting disease progression according to rs1801157. We conclude that CXCL12 rs1801157 is not a general susceptibility factor for colon or rectal cancer in the Spanish population. The deviation attributed to females may suggest a putative role of CXCL12 in rectal cancer aetiology. However, these results need further confirmation, since they do not pass multiple testing corrections. Whenever an association study is reported, statistical power is frequently questioned. For this reason, we performed the corresponding studies taking into account the case/control ratio used, our case sample size and the minor allele frequency of rs1801157 in our control population. Our study was 80% powered to detect an odds ratio >1.53, and 90% powered for an odds ratio >1.63.

Nevertheless, the absence of an association with CRC risk does not exclude the possibility that rs1801157 may influence cancer progression. In fact, it has been reported that circulating levels of CXCL12 are significantly lower among CRC patients when compared to healthy people, and this feature also correlates with the tumor stage (24). For this reason we performed different analyses to elucidate whether rs1801157 may be conditioning the tumor aggressiveness profile. According to our results, no correlation has been found among the typical progression markers such as histological grade, tumor size or metastatic profile. For this reason, we performed the corresponding studies in order to test whether rs1801157 is associated to a differential prognosis. Kaplan-Meier analysis revealed that those patients harbouring the

GA+AA) exhibit a similar progression than those ous for GG (Mantel-Cox log-rank test, p=0.63). It must be stated that we do not question the role that CXCR12 might play in CRC aetiology and/or in metastasis formation. However, according to our results rs1801157 appears not to be linked to this effect. The genetic data presented so far suggest that CXCL12 rs1801157 does not play a role in cancer risk, but a second independent progression analysis is necessary to confirm this hypothesis.

Acknowledgments

We are deeply grateful to CRC patients and controls for their participation in this study and Dr Carlos San Roman for patient recruitment. We also thank Ana Salinas, Juan Velasco and Maria del Carmen Rivero for their excellent technical support during this study. JLR holds a Torres Quevedo grant (PTQ04-1-0006) from the Spanish Ministerio de Educación y Ciencia.

References

1. Potter JD: Related colorectal cancer: molecules and populations. J Natl Cancer Inst 91: 916-932, 1999.
2. Lu H, Ouyang W and Huang C: Inflammation, a key event in cancer development. Mol Cancer Res 4: 221-233, 2006.
3. Yan L, Anderson GM, DeWitte M and Nakada MT: Therapeutic potential of cytokine and chemokine antagonists in cancer therapy. Eur J Cancer 6: 793-802, 2006.
4. Lee WP, Tai DI, Lan KH, Li AF, Hsu HC, Lin EJ, Lin YP, Sheu ML, Li CP, Chang FY, Chao Y, Yen SH and Lee SD: The -251T allele of the interleukin-8 promoter is associated with increased risk of gastric carcinoma featuring diffuse-type histopathology in Chinese population. Clin Cancer Res 18: 6431-6441, 2005.
5. Duell EJ, Casella DP, Burk RD, Kelsey KT and Holly EA: Inflammation, genetic polymorphisms in proinflammatory genes TNF-A, RANTES, and CCR5, and risk of pancreatic adenocarcinoma. Cancer Epidemiol Biomarkers Prev 4: 726-731, 2006.
6. Manes S, Mira E, Colomer R, Montero S, Real LM, Gomez-Mouton C, Jimenez-Baranda S, Garzon A, Lacalle RA, Harshman K, Ruiz A and Martinez-A C: CCR5 expression influences the progression of human breast cancer in a p53-dependent manner. J Exp Med 198: 1381-1389, 2003.
7. Burger JA and Kipps TJ: CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. Blood 107: 1761-1767, 2006.
8. Campbell JJ, Hedrick J, Zlotnik A, Siani MA, Thompson DA and Butcher EC: Chemokines and the arrest of lymphocytes rolling under flow conditions. Science 279: 381-384, 1998.
9. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL and Weinberg RA: Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 121: 335-348, 2005.
10. Wehler T, Wolfert F, Schimanski CC, Gockel I, Herr W, Biesterfeld S, Seifert JK, Adwan H, Berger MR, Junginger T, Galle PR and Moehler M: Strong expression of chemokine receptor CXCR4 by pancreatic cancer correlates with advanced disease. Oncol Rep 6: 1159-1164, 2006.
11. Rubie C, Frick VO, Wagner M, Weber C, Kruse B, Kempf K, Konig J, Rau B and Schilling M: Chemokine expression in hepatocellular carcinoma versus colorectal liver metastases. World J Gastroenterol 12: 6627-6633, 2006.
12. Zagzag D, Krishnamachary B, Yee H, Okuyama H, Chiriboga L, Ali MA, Melamed J and Semenza GL: Stromal cell-derived factor-1alpha and CXCR4 expression in hemangioblastoma and clear cell-renal cell carcinoma: von Hippel-Lindau loss-of-function induces expression of a ligand and its receptor. Cancer Res 65: 6178-6188, 2005.
13. Staller P, Sulitkova J, Lisztwan J, Moch H, Oakeley EJ and Krek W: Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumour suppressor pVHL. Nature 425: 307-311, 2003.
14. Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E and Zlotnik A: Involvement of chemokine receptors in breast cancer metastasis. Nature 410: 50-56, 2001.
15. Brand S, Dambacher J, Beigel F, Olszak T, Diebold J, Otte JM, Goke B and Eichhorst ST: CXCR4 and CXCL12 are inversely expressed in colorectal cancer cells and modulate cancer cell migration, invasion and MMP-9 activation. Exp Cell Res 310: 117-130, 2005.
16. Zeelenberg IS, Ruuls-Van Stalle L and Roos E: The chemokine receptor CXCR4 is required for outgrowth of colon carcinoma micrometastases. Cancer Res 63: 3833-3839, 2003.
17. Chinni SR, Sivalogan S, Dong Z, Filho JC, Deng X, Bonfil RD and Cher ML: CXCL12/CXCR4 signaling activates Akt-1 and MMP-9 expression in prostate cancer cells: the role of bone microenvironment-associated CXCL12. Prostate 66: 32-48, 2006.
18. Gockel I, Schimanski CC, Heinrich C, Wehler T, Frerichs K, Drescher D, von Langsdorff C, Domeyer M, Biesterfeld S, Galle PR, Junginger T and Moehler M: Expression of chemokine receptor CXCR4 in esophageal squamous cell and adenocarcinoma. BMC Cancer 6: 290, 2006.
19. Winkler C, Modi W, Smith MW, *et al*: Genetic restriction of AIDS pathogenesis by an SDF-1 chemokine gene variant. Science 279: 389-393, 1998.
20. Gerli G, Vanelli C, Turri O, Erario M, Gardellini A, Pugliano M and Biondi ML: SDF1-3'A gene polymorphism is associated with chronic myeloproliferative disease and thrombotic events. Clin Chem 51: 2411-2414, 2005.
21. Razmkhah M, Talei AR, Doroudchi M, Khalili-Azad T and Ghaderi A: Stromal cell-derived factor-1 (SDF-1) alleles and susceptibility to breast carcinoma. Cancer Lett 225: 261-266, 2005.
22. Razmkhah M, Doroudchi M, Ghayumi SM, Erfani N and Ghaderi A: Stromal cell-derived factor-1 (SDF-1) gene and susceptibility of Iranian patients with lung cancer. Lung Cancer 49: 311-315, 2005.
23. Ide A, Kawasaki E, Abiru N, Sun F, Fukushima T, Takahashi R, Kuwahara H, Fujita N, Kita A, Oshima K, Uotani S, Yamasaki H, Yamaguchi Y, Kawabata Y, Fujisawa T, Ikegami H and Eguchi K: Stromal-cell derived factor-1 chemokine gene variant is associated with type 1 diabetes age at onset in Japanese population. Hum Immunol 64: 973-978, 2003.
24. Dimberg J, Hugander A, Lofgren S and Wagsater D: Polymorphism and circulating levels of the chemokine CXCL12 in colorectal cancer patients. Int J Mol Med 19: 11-15, 2007.
25. Royo JL, Ruiz A, Borrego S, Rubio A, Sanchez B, Nunez-Roldan A, Lissen E and Antinolo G: Fluorescence resonance energy transfer analysis of CCR-V64I and SDF1-3'a polymorphisms: prevalence in southern Spain hiv type 1+ cohort and noninfected population. AIDS Res Hum Retroviruses 17: 663-666, 2001.
26. Rothman KJ: Epidemiologic analysis with programmable calculator. US NIH publication no. 79-1649.
27. Sasieni PD: From genotypes to genes: doubling the sample size. Biometrics 53: 1253-1261, 1997.
28. Juarez J, Bendall L and Bradstock K: Chemokines and their receptors as therapeutic targets: the role of the SDF-1/CXCR4 axis. Curr Pharm Res 10: 1245-1259, 2004.
29. Tamamura H, Tsutsumi H and Fujii N: The chemokine receptor CXCR4 as a therapeutic target for several diseases. Mini Rev Med Chem 6: 989-995, 2006.