

# Association between *CDKN1A* Ser31Arg and C20T Gene Polymorphisms and Colorectal Cancer Risk and Prognosis

CANAN CACINA<sup>1</sup>, İLHAN YAYLIM-ERALTAN<sup>1</sup>, SOYKAN ARİKAN<sup>2</sup>,  
ESRA KAYTAN SAGLAM<sup>3</sup>, UMİT ZEYBEK<sup>1</sup> and TURGAY ISBİR<sup>1</sup>

<sup>1</sup>Institute of Experimental Medicine, Department of Molecular Medicine, Istanbul University, İstanbul, Turkey;

<sup>2</sup>Istanbul Education and Research Hospital Surgery Clinic, İstanbul, Turkey;

<sup>3</sup>Yeditepe University, Medical Faculty Department of Medical Biology, İstanbul, Turkey

**Abstract.** Background: *CDKN1A* ( $p21^{WAF1/CIP1}$ ) plays an important role in cell cycle regulation. Somatic alterations in genes which regulate cell division have been shown to be related to different types of cancer prognosis and survival. The purpose of this study was to investigate the effect of the *CDKN1A* Ser31Arg and C20T gene polymorphisms in Turkish patients with colorectal cancer. Patients and Methods: *CDKN1A* Ser/Arg and C20T polymorphisms were studied in 53 patients with colorectal cancer and 64 healthy controls. Genomic DNA was amplified by polymerase chain reaction (PCR) and genotypes were determined by the restriction fragment length polymorphism (RFLP) method. Results: There were statistically significant differences in the distribution of *CDKN1A* Ser/Arg genotypes and allele frequencies between colorectal cancer patients and healthy controls ( $p=0.040$  and  $p=0.01$ , respectively). *CDKN1A* C20T genotype frequency did not show any significant differences between patients and controls. We combined the results for C20T and Ser31Arg polymorphisms and observed that a lower risk of colorectal cancer was associated with CT/SerArg combined genotypes compared to controls and this difference was statistically significant ( $p=0.024$ ; odds ratio (OR)=0.322, 95% confidence interval (CI)=0.114-0.912). C20T C allele and SerSer genotypes significantly increased risk compared to other combined genotypes ( $p=0.034$ ; OR=1.265, 95% CI=1.020-1.569). Conclusion: The results of present study demonstrated that, potentially, *CDKN1A* functional polymorphisms may contribute to the risk of colorectal cancer in Turkish.

Correspondence to: Prof. Dr. Turgay Isbir, Yeditepe University, Medical Faculty Department of Medical Biology, İstanbul, Turkey, İnönü Mah. Kayışdağı Cad. 26 Ağustos Yerleşimi, 34755 Kadıköy - İstanbul. Tel/Fax: +90216 5780000, e-mail: tisbir@superonline.com

Key Words: *CDKN1A*, polymorphism, cell cycle.

Molecular mechanisms in colorectal cancer initiation and progression consist of the imbalance between cell growth, apoptosis, differentiation and are associated with the accumulation of genetic alterations over a period of many years (1, 2). Somatic alterations of genes involved in the control of progression from the G<sub>1</sub> to the S phase of the cell cycle, including the cyclins, cyclin dependent kinases (CDKs), and CDK inhibitors, are common occurrences in neoplastic development for multiple tumor types (3, 4). Cyclin proteins and CDKs form complexes which regulate cell growth through cell-cycle control and CDK inhibitors inhibit the kinase activities of these complexes and arrest cell-cycle transitions (5, 7).

*CDKN1A* belongs to the CIP/KIP family, which includes p27 (8) and p57 (9, 10). It is located on chromosome 6p21.2 and encodes a 21-kDa protein (11). CDK inhibitor *CDKN1A* plays important roles in modulating cellular proliferation, differentiation and apoptosis (8). *CDKN1A* binds to cyclin complexes to modulate cell cycle progression, preventing the division of DNA damaged cells by inhibiting the function of CDKs (12, 13). Expression of *CDKN1A* is up-regulated by p53 in response to DNA damage to induce cell cycle arrest at the G<sub>1</sub> checkpoint (8). Most studies have focused on polymorphisms of *CDKN1A* and the high-risk genotypes have been reported to be associated with different types of cancer (14-18).

Several single nucleotide polymorphisms (SNPs) are described in *CDKN1A* including: a serine to arginine (Ser/Arg) substitution at codon 31; and a single-base substitution of C to T at a point 20 bp 3 of the stop codon (16). Studies of some cancer types have noted associations between the *CDKN1A*<sup>WAF1</sup>, codon 31 polymorphism and cancer risk. These include prostate and cervical adenoma, cancer of the head and neck, and some types of lung cancer (16, 19-21). It is reported that the two polymorphisms are in strong linkage disequilibrium, and it has been hypothesized that each may cause functional changes of *CDKN1A* (19).

The aim of the present study was to investigate whether *CDKN1A* Ser31Arg and C20T polymorphisms are involved in the development of sporadic colorectal cancer (CRC) and its prognosis.

## Patients and Methods

**Patient selection and clinical investigation.** The present study was approved by the Ethical Committee of Istanbul University, The Istanbul Faculty of Medicine (No: 1746).

This study includes 53 colorectal cancer patients who were treated at Istanbul Education and Research Hospital Surgery Clinic and Istanbul University, Institute of Oncology, Department of Radiation Oncology, in 2007 and 2008. The mean age of colorectal cancer patients was 61.57±14.34 years. The diagnoses of the patients were determined by colonoscopic radiological and operative findings and confirmed by pathological examination. The blood samples were collected from the patients before any treatment had been started (chemotherapy or radiotherapy). A total of 64 healthy and ethnically matched blood donors with a mean age of 57.18±11.52 years served as controls. Informed consent was given by all participants.

**DNA isolation.** Blood specimens were collected in tubes containing EDTA and DNA samples were extracted from whole blood by a salting-out procedure (22).

*CDKN1A* (Ser/Arg polymorphism) genotypes were determined using a polymerase chain reaction (PCR) amplification and digestion assay. A 272-bp PCR amplification product of Ser31Arg was generated using the primers 5'-GTC AGA ACC GGC TGG GGA TG- 3' and 5'-CTC CTC CCA ACT CAT CCC GG-3'. The reaction mix contained 50-100 ng genomic DNA amplified with 1x PCR buffer, 0.2 mM of each dNTP, 3 mM MgCl<sub>2</sub>, 0.2 mM of each primer and 0.5 U of Taq polymerase (MBI Fermentas, Lithuania) in a 25 ml reaction volume. After denaturing the DNA for 5 min at 94°C, the reaction mixture was subject to 35 cycles of denaturing for 30 s at 94°C, 30 s annealing at 60°C and 72°C for 30 s. The final extension step was at 72°C for 7 min. The PCR products were digested with *BlnI* restriction enzyme (MBI Fermentas) at 37°C for 16 h followed by electrophoresis in 2% agarose gel containing ethidium bromide. The Arg/Arg genotype (homozygote of infrequent allele) lacks a *BlnI* site and shows only one band of 272 bp. The Ser/Ser genotype (homozygote of common allele) generates two fragments of 183 and 89 bp. The heterozygote displays three fragments of 272, 183 and 89 bp, designated as Ser/Arg (23).

*CDKN1A* (C20T) genotypes were determined using a PCR amplification. PCR amplification product of C20T was generated using the primers 5'-TCCAAGAGGAAGCCCTAATC-3' and 5'-AAAGGAGAACACGGGATGAG-3'. For detection of the C20T RFLP, 50-100 ng genomic DNA were amplified with 1x PCR buffer, 0.2 mM of each dNTP, 3 mM MgCl<sub>2</sub>, 0.2 mM of each primer and 0.5 U of Taq polymerase (MBI Fermentas) in a 25 µl reaction volume. The initial denaturation step of 94°C for 5 min followed by 35 cycles of 94°C for 15 s, 64.8°C for 30 s, and 72°C for 1 min and a further 72 for 7 min. The PCR product was digested with *PstI* restriction enzyme (MBI Fermentas) at 37°C for 16 hours and was electrophoresed in 2% agarose gels and stained with ethidium bromide. C20T genotypes were determined as CC (258, 39 bp), CT (297, 258, 39 bp), or TT (297 bp) (17, 23, 24).

Table I. Characteristics of patients with colorectal cancer.

	N	%
No. of patients	53	
Smoking status		
Yes	5	9.4
No	48	90.6
Alcohol consumption		
Yes	15	28.3
No	38	71.7
Family history of any kind of cancer		
Yes	1	1.9
No	52	98.1
Tumor localization		
Left colon	7	20
Right colon	5	14.3
Transverse colon	3	8.6
Rectum	15	42.9
Sigmoid	5	14.3
T stage		
T1	3	8.6
T2	5	14.3
T3	22	62.9
T4	5	14.3
Lymph node status		
N0	19	54.3
N1	10	28.6
N2	5	14.3
N3	1	2.8
Distant metastasis (+)	4	11.8

The pathological data of eighteen patients were unavailable.

Fisher's exact test was used to calculate odds ratio (OR) with 95% confidence interval (CI) to determine whether *CDKN1A* genotypes were associated with the disease. In addition, patients were stratified by age at presentation and aggressiveness of disease to determine whether an association existed between genotype and age of onset and development of colorectal cancer.

## Results

Selected characteristics of 53 colorectal cancer cases and 64 cancer-free controls included in our study are summarized in Table I. The genotype distributions of *CDKN1A* Ser31Arg and C20T in our patients and controls are shown Table II. With the use of the  $\chi^2$  test, the difference in the distribution of three *CDKN1A* Ser31Arg genotypes and allelic frequencies between colorectal cancer patients and controls were found to be statistically significant, with the Ser allele and Ser/Ser genotype being more frequent in patients. ( $p=0.040$  and  $p=0.01$ , respectively). There were no significant differences between the cases and the controls for *CDKN1A* C20T genotypes and allelic frequencies. Our results indicate that individuals carrying the SerArg genotype have a decrease in the risk for development of colorectal cancer (OR=0.517, 95% CI=0.271-0.985). When we

Table II. Genotype and allele frequencies for *CDKN1A* in colorectal cancer patients and controls.

Genotype	Patients	%	Controls	%	<i>p</i> -Value	
<i>CDKN1A</i> Ser31Arg (C>A)						
SerSer	42	79.2	36	56.3	0.029	
SerArg	10	18.9	24	37.5		
ArgArg	1	1.9	4	6.3		
Ser	94	88.7	96	75		0.008
Arg	12	11.3	32	25		
<i>CDKN1A</i> C20T						
TT	4	7.5	6	9.4	0.588	
CT	25	47.2	35	54.7		
CC	24	45.3	23	35.9		
T	33	31.1	47	36.7		
C	73	68.9	81	63.3		0.369

*p*-Value obtained by Chi-square test.

examined the combined effect of these two genotype variants on colorectal cancer risk, we found that a lower risk of colorectal cancer was associated with the CT/SerArg combined genotype compared to controls, and this difference was statistically significant ( $p=0.024$ ; OR=0.322, 95% CI=0.114-0.912). *CDKN1A* CT and SerSer was the most common genotype in controls and in patients with colorectal cancer, and this combined genotype was observed more frequently in colorectal cancer patients than controls (OR=1.271, 95% CI=0.762-2.120); Table II). When we combined the results for C20T and Ser31Arg polymorphism, we also found a significant increased risk for those with C20T C allele and SerSer genotype compared to other combined genotypes ( $p=0.034$ ; OR=1.265, 95% CI=1.020-1.569).

In addition, we performed stratification analyses by colorectal cancer stage, histological subtype and other demographic variables; however, the risk of colorectal cancer associated with the combined *CDKN1A* Ser31Arg and C20T genotypes were not significantly different among individuals with different stage and histological subtypes, nor among age at diagnosis, with or without family history of cancer and smoking status (data not shown).

## Discussion

*CDKN1A* has been characterized as a putative tumor suppressor gene (25). Decreased protein levels of *CDKN1A* were observed in a subset of cancer types including breast, and laryngeal cancer, indicating the importance of this gene in tumorigenesis (26, 27). Allelic frequency patterns of *CDKN1A* Ser31Arg (C>A) and C20T polymorphisms vary greatly between different populations. The frequency of the Ser allele of *CDKN1A* in Caucasians was found to be 0.1, consistent with published reports, but the allelic frequencies

Table III. Distribution of the estimated combined genotype frequencies for *CDKN1A* gene in cases and controls.

Combined genotype (C20T and Ser31Arg)	Patients N (%)	Controls N (%)	<i>p</i> -Value
CC/SerSer	19 (35.8)	15 (23.4)	0.141
CC/SerArg	5 (9.4)	7 (10.9)	0.790
CC/ArgArg	0 (0)	1 (1.6)	1.000
CT/SerSer	20 (37.7)	19 (29.7)	0.358
CT/SerArg	4 (7.5)	15 (23.4)	0.024*
CT/ArgArg	1 (1.9)	1 (1.6)	1.000
TT/SerSer	3 (5.7)	2 (3.1)	0.657
TT/SerArg	1 (1.9)	2 (3.1)	1.000
TT/ArgArg	0 (0)	2 (3.1)	0.500

*p*-Value computed between selected genotype versus all others genotypes in corresponding group, \* $p<0.05$ .

of *CDKN1A* by race were found to vary (19, 28). It was reported that the Ser allele frequencies for African-Americans and Latinas are 0.27 and 0.24, respectively (29). The Ser allele frequency in our study was found to be 0.76. The frequency of the *CDKN1A* Arg allele was 0.06 in Caucasian (30), around 0.5 in Asian (15, 20) and 0.459 in Chinese populations (24). The frequency of the *CDKN1A* Arg and C20T variant homozygote in our study was 0.24 and 0.37, respectively.

A number of molecular epidemiological studies have examined the effects of *CDKN1A* polymorphisms on the risk of different types of cancer. Several studies showed that the *CDKN1A* Ser31Arg polymorphism has been associated with cancer of breast and colorectum (29, 31).

The alteration at codon 31 exchanges a serine, an uncharged polar amino acid with a single hydroxyl side chain, for an arginine, which is a basic, positively charged amino acid with a seven-membered side chain. These alterations suggest that this change may create a phenotypic variant of the *CDKN1A* protein (15). In a single locus analysis, we found that *CDKN1A* SerArg heterozygotes, but not the homozygotes, had a significantly lower risk for development of colorectal cancer. Molecular heterosis has been described in which individuals heterozygous for a specific genetic polymorphism show stronger effects than those homozygous for either allele (32). Although the reason for a lower risk associated with the Ser31Arg variant heterozygote remains unclear, it is possible that this heterozygosity may prevent potential imbalance of the protein function. It is known that the Ser/Arg polymorphism at codon 31 is located in a highly conserved region of the *CDKN1A* gene and encodes a probable DNA-binding zinc-finger domain, causing functional changes to *CDKN1A* protein (23).

Mousses *et al.* (16) reported that the *CDKN1A* Ser31Arg locus was in strong linkage disequilibrium with the 3'UTR

C20T locus, which may effect mRNA stability and was suggested to be related to the risk of prostate cancer and squamous cell carcinoma of the head and neck (16, 17). We also found an decreased risk in individuals with CT/SerArg combined genotype for colorectal cancer. The results presented here indicate that codon 31 polymorphism alone is not sufficient to predict colorectal cancer risk. In contrast to individual SNPs, whether a combination of *CDKN1A* C20T C allele and homozygous SerSer genotype improves the prediction of colorectal cancer risk interested us. When this comparison was carried out between our colorectal cancer patients and controls, we found a significant increased risk for these with C20T C allele and SerSer genotype compared to other combined genotypes.

We observed that the combined genotype significantly increased the probability of developing colorectal cancer. Our results demonstrated that *CDKN1A* functional polymorphisms may potentially contribute to risk of colorectal cancer in the Turkish population. Additional investigations into how different genotypes may affect the functions of *CDKN1A* proteins will provide a better strategy for identifying these at risk of colorectal cancer.

## Acknowledgements

The present work was supported by the Research Fund of Istanbul University. Project No. 1746.

## References

- Fujiwara T, Stolker JM, Watanabe T, Rashid A, Longo P, Eshleman JR, Booker S, Lynch HT, Jass JR, Green JS, Kim H, Jen J, Vogelstein B and Hamilton SR: Accumulated clonal genetic alterations in familial and sporadic colorectal carcinomas with widespread instability in microsatellite sequences. *Am J Pathol* 153: 1063-1078, 1998.
- Hao XP, Du MQ, Bishop AE and Talbot IC: Imbalance between proliferation and apoptosis in the development of colorectal carcinoma. *Virchows Arch* 433: 523-527, 1998.
- Barbieri F, Cagnoli M, Ragni N, Foglia G, Bruzzo C, Pedullà F and Alama A: Increased cyclin D1 expression is associated with features of malignancy and disease recurrence in ovarian tumors. *Clin Cancer Res* 5: 1837-1842, 1999.
- Shigemasa K, Tanimoto H, Parham GP, Parmley TH, Ohama K and O'Brien TJ: Cyclin D1 overexpression and *p53* mutation status in epithelial ovarian cancer. *J Soc Gynecol Investig* 6: 102-108, 1999.
- Cordon-Cardo C: Mutations of cell cycle regulators. Biological and clinical implications for human neoplasia. *Am J Pathol* 147: 545-560, 1995.
- Hunter T and Pines J: Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age. *Cell* 79: 573-582, 1994.
- MacLachlan TK, Sang N and Giordano A: Cyclins, cyclin-dependent kinases and cdk inhibitors: implications in cell cycle control and cancer. *Crit Rev Eukaryot Gene Expr* 5: 127-156, 1995.
- Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R and Beach D: p21 is a universal inhibitor of cyclin kinases. *Nature* 366: 701-704, 1993.
- Lee MH, Reynisdottir I and Massague J: Cloning of *p57/KIP2*, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes Dev* 9: 639-649, 1995.
- Matsuoka S, Edwards MC, Bai C, Parker S, Zhang P, Baldini A, Harper JW and Elledge SJ: *p57/KIP2*, a structurally distinct member of the *p21/CIP1* Cdk inhibitor family is a candidate tumor suppressor gene. *Genes Dev* 9: 650-662, 1995.
- el Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin DM, Mercer WE, Kinzler KW and Vogelstein B: WAF1, a potential mediator of p53 tumor suppression. *Cell* 75: 817-825, 1993.
- Harper JW, Adami GR, Wei N, Keyomarsi K and Elledge SJ: The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G<sub>1</sub> cyclin-dependent kinases. *Cell* 75: 805-816, 1993.
- Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW and Vogelstein B: Requirement for p53 and p21 to sustain G<sub>2</sub> arrest after DNA damage. *Science* 282: 1497-1501, 1998.
- Facher EA, Becich MJ, Deka A and Claw JC: Association between human cancer and two polymorphisms occurring to get her in the *p21<sup>Waf1/Cip1</sup>* cyclin-dependent kinase inhibitor gene. *Cancer* 79: 2424-2429, 1997.
- Lukas J, Groshen S, Saffari B, Niu N, Reles A, Wen WH, Felix J, Jones LA, Hall FL and Press MF: *WAF1/Cip1* gene polymorphism and expression in carcinomas of the breast, ovary, and endometrium. *Am J Pathol* 150: 167-175, 1997.
- Mousses S, Ozcelik H, Lee PD, Malkin D, Bull SB and Andrulis IL: Two variants of the *CIP/WAF1* gene occur together and are associated with human cancer. *Hum Mol Genet* 4: 1089-1092, 1995.
- Kibel AS, Suarez BK, Belani J, Oh J, Webster R, Brophy-Ebbers M, Guo C, Catalona WJ, Picus J and Goodfellow PJ: *CDKN1A* and *CDKN1B* polymorphisms and risk of advanced prostate carcinoma. *Cancer Res* 63(9): 2033-2036, 2003.
- Rodrigues FC, Kawasaki-Oyama RS, Fo JF, Ukuyama EE, Antonio JR, Bozola AR, Romeiro JG, Rahal P and Tajara EH: Analysis of *CDKN1A* polymorphisms: markers of cancer susceptibility? *Cancer Genet Cytogenet* 142: 92-98, 2003.
- Sjalander A, Birgander R, Rannug A, Alexandrie AK, Tornling G and Beckman G: Association between the *p21* codon 31 A1(Arg) allele and lung cancer. *Hum Hered* 46: 221-225, 1996.
- Hachiya T, Kuriaki Y, Ueoka Y, Nishida J, Kato K and Wake N: *WAF1* genotype and endometrial cancer susceptibility. *Gynecol Oncol* 72: 187- 92, 1999.
- Milner BJ, Brown I, Garba H, Kitchener HC, Parkin DE and Haites NE: A protective role for common *p21<sup>WAF1/CIP1</sup>* polymorphism in human ovarian cancer. *Int J Oncol* 15: 117-119, 1999.
- Miller SA, Dykes DD and Polesky HF: A simple salting-out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 16(3): 1215, 1988.
- Ressiniotis T, Griffiths GP, Keers MF, Chinnery PA and Birch M: A polymorphism at codon 31 of gene *p21* is not associated with primary open angle glaucoma in Caucasians. *BMC Ophthalmology* 5: 5, 2005.
- Ma H, Jin G, Hu Z, Zhai X, Chen W, Wang S, Wabg X, Qin J, Gao J, Liu J, Wang X, Wei Q and Shen H: Variant genotypes of *CDKN1A* and *CDKN1B* are associated with an increased risk of breast cancer in Chinese women. *Int J Cancer* 119: 2173-2178, 2006.

- 25 Shiohara M, el-Deiry WS, Wada M, Nakamaki T, Takeuchi S, Yang R, Chen DL, Vogelstein B and Koeffler HP: Absence of *WAF1* mutations in a variety of human malignancies. *Blood* 84: 3781-3784, 1994.
- 26 Jiang M, Shao ZM, Wu J, Lu JS, Yu LM, Yuan JD, Han QX, Shen ZZ and Fontana JA: p21/waf1/cip1 and mdm-2 expression in breast carcinoma patients as related to prognosis. *Int J Cancer* 74: 529-534, 1997.
- 27 Pruneri G, Pignatoro L, Carboni N, Bufa R, Di Finizio D, Cesana BM and Neri A: Clinical relevance of expression of the CIP/KIP cell-cycle inhibitors p21 and p27 in laryngeal cancer. *J Clin Oncol* 17: 3150-3159, 1999.
- 28 Birgander R, Sjalander A, Saha N, Spitsyn V, Beckman L and Beckman G: The codon 31 polymorphin of the p53-inducible gene *p21* shows distict differences between major ethnic groups. *Hum Hered* 46: 148-154, 1996.
- 29 Kesheva C, Frye BL, Wolff MS, McCanlies EC and Weston A: *Waf-1 (p21)* and *p53* polymorphisms in breast cancer. *Cancer Epidemiol Biomarkers Prev* 11: 127-130, 2002.
- 30 Chedid M, Michieli P, Lengel C, Huppi K and Givol D: A single nucleotide substitution at codon 31 (Ser/Arg) defines a polymorphism in a highly conserved region of the p53-inducible gene *WAF/CIP1*. *Oncogene* 9: 3021-3024, 1994.
- 31 Li YJ, Laurent-Puig P, Salmon RJ, Thomas G and Hamelin R: Polymorphisms and probable lack of mutation in the *WAF1-CIP1* gene in colorectal cancer. *Oncogene* 10: 599-601, 1995.
- 32 Comings DE and MacMurray JP: Molecular heterosis: a review. *Mol Genet Metab* 71: 19-31, 2000.

*Received August 30, 2009*  
*Revised February 2, 2010*  
*Accepted February 9, 2010*