

## Are Factor V and Prothrombin Mutations Associated with Increased Risk of Oral Cancer?

ELEFThERIOS VAIRAKTARIS<sup>1</sup>, CHRISTOS YAPIJAKIS<sup>1,2</sup>, JÖRG WILTFANG<sup>3</sup>, JUTTA RIES<sup>3</sup>, ANTONIS VYLLIOTIS<sup>1,2</sup>, SPYRIDOULA DERKA<sup>1</sup>, STAVROS VASILIOU<sup>1</sup> and FRIEDRICH W. NEUKAM<sup>3</sup>

<sup>1</sup>Department of Maxillofacial Surgery, University of Athens Medical School, Vas. Sofias 93 & Dim. Soutsou 1, Athens 11521;

<sup>2</sup>Department of Molecular Biology and Genetics, "Bioerevna" Medical Center, Marni 4, Athens 10433, Greece

<sup>3</sup>Department of Maxillofacial Surgery, Universität Erlangen, Klinik und Poliklinik für Mund-, Kiefer-, Gesichtschirurgie, Glueckstrasse 11, Erlangen D-91054, Nürnberg, Germany

**Abstract.** *Background: Methylene tetrahydrofolate reductase is associated with pathogenesis of both thrombosis and oral cancer. Therefore, a search for a similar association of other thrombosis-related factors with oral cancer is justified. Patients and Methods: In order to investigate whether the coagulation factor V Leiden and prothrombin G20210A mutations increase the risk of oral cancer, we searched for these mutant alleles by RFLP analysis in DNA samples of 102 patients with oral cancer and 120 healthy controls. Results: Neither the Leiden nor the G20210A alleles were found in statistically different frequencies in the two groups. In addition, no statistical difference was observed in parameters such as sex, age and positive family history for cancer. Nevertheless, a significant difference was observed for Leiden in patients with a positive history for thrombophilia ( $p < 0.001$ ). Conclusion: There seems to be no association of prothrombin, and possibly a minor contribution of factor V, in oncogenesis in the oral region.*

Oral cancer is mainly caused by environmental factors and mutations in several genes (1, 2). Moreover, oral cancer, as with any type of cancer, has an inseparable association with the coagulation system. The coagulation system may make a substantial contribution to tumor angiogenesis, which represents an imbalance in the normal mechanisms that allow organized healing after injury (3). Manipulations of the coagulation system and angiogenesis pathways may

minimize both the neoangiogenesis essential for tumor growth and associated thromboembolic complications (3). However, since surgery is the primary treatment for most cancers, the angiogenesis of wound healing and hemostatic competence must be maintained (3). Therefore, due to these complex interactions between the coagulation system and the angiogenic process that occur in cancer growth, the study of genes associated with the coagulation system looks promising (3). One of these genes is the *MTHFR* gene, which encodes the methylene tetrahydrofolate reductase enzyme and has been associated with increased risk of several cancers, in addition to oral cancer (4-10). *MTHFR* is responsible for the circulating form of folate, 5-methyl-tetrahydrofolate, which converts methionine to S-adenosylmethanone, *i.e.* the universal methyl donor for various intracellular methylation reactions, particularly DNA methylation (11, 12). A germ-line mutation has been identified in the *MTHFR* gene at nucleotide 677 (C677T) and the variant genotype is associated with an increased thermolability and significantly diminished specific activity of the enzyme (11, 12). It has recently been shown that the C677T genotype causes elevation in homocysteine levels compared to the normal genotype (11-14). Homozygotes for the mutation account for 8-8.5% of the general population, and they are known to have a higher risk of thrombotic events (thrombophilia) (12-16). They also display a higher risk for development of oral cancer, according to some reports (8-10).

In light of the established contribution of one genetic factor associated both with thrombophilia and oral cancer, the investigation of other such thrombophilia- predisposing factors and their possible association with oncogenesis in the oral region is justified. Two such coagulation factors, with common mutations in the general population, are factor V and factor II (prothrombin). It is interesting that there have already been reports of an association between

*Correspondence to:* Christos Yapijakis, DMD,MS,PhD, Department of Molecular Biology and Genetics, "Bioerevna" Medical Center, Marni 4, Athens 10433, Greece. Tel: +30-210-8213444, Fax: +30-210-8811243, e-mail: cyapijakis\_ua\_gr@yahoo.com

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factor V Leiden mutation and prothrombin G20210A mutation with other types of cancer, such as gastrointestinal carcinoma, lung carcinoma, lymphoblastic leukemia *etc.* (4, 7, 17-20).

Mutant factor V Leiden is responsible for resistance to protein C, a fact associated with clinical hypercoagulability (21, 22). This missense mutation causes an arginine to glutamine substitution in one of the protein's cleavage sites and renders activated factor V relatively resistant to cleavage and, thus, inactivation, by activated protein C (21). This mutation is now recognized as the most common cause of heritable thrombophilia (22). Prothrombin, on the other hand, is the precursor molecule of thrombin, which activates factors V and VIII and converts fibrinogen to fibrin. A G to A transition at nucleotide position 20210, in the 3'-untranslated region of the gene, is associated with an increased risk of venous thrombosis (23, 24). Patients with the G20210A allele have significantly higher levels of plasma prothrombin, which is believed to mediate the procoagulant effect (23, 24). In European populations, including Greeks and Germans, each one of the two mutations display frequencies of 4-5% (15, 22-25).

In order to investigate whether the Leiden and G20210A alleles increase the risk of oral cancer, we searched for the frequencies of these mutations in DNA samples of 102 patients with oral cancer, in comparison to 120 healthy controls of similar ethnicity, age and sex.

## Patients and Methods

The subjects under study included 222 unrelated individuals of Greek (N=111) and German (N=111) origin, namely 102 patients with oral squamous cell carcinoma and 120 healthy controls with matched ethnicity, sex and age. The patients had an age range of 40-83 years (52.1+/-7.2 years, median 58 years) and were mostly men (N=90). The controls had comparable age (range 40-82; 51.5 +/- 5.5 years, median 58 years) and sex ratio (N=102 men).

Patients who had developed oral cancer and had been operated recently or up to 10 years previously were included in this study. In addition to clinical presentation, a biopsy with pathological diagnosis of tumor was needed in order for a patient to be included in the study.

For each patient, a family history of cancer and/or thrombophilia was collected after informed consent. Forty-two patients (41.2%) had one or two first-degree relatives with any type of cancer. Their age range (median 58 years) was not significantly different than the whole group. Furthermore, twenty-six patients (25.5%) had one or two first-degree relatives with idiopathic thrombosis, with an earlier age range (median 52 years), but again with no statistical difference. Fourteen patients (13.7%) had a positive family history for both cancer and thrombophilia (median 48 years).

Blood samples were collected from both the patient and the control groups. All studied individuals were fully informed about the potential meaning of test results and willingly participated in the study. The samples were assigned a number and examined

blindly. DNA was extracted from blood with the use of the Nucleon™ kit (Amersham). Molecular analysis for the Leiden mutation in the factor V gene and the G20210A mutation in the prothrombin gene was performed as previously described (20, 23). Briefly, it involved a combination of PCR and digestion with restriction endonuclease Taq I, followed by agarose gel electrophoresis analysis. The presence of each mutation alters a Taq I recognition site, therefore the PCR product is not cleaved by the enzyme. After Taq I treatment, the PCR product of 175 bp for factor V and of 118 bp for prothrombin is seen on electrophoresis as two fragments (157+18 bp and 98+10 bp, respectively) when a normal allele is present, and intact when a mutant allele exists.

The frequencies of alleles and genotypes found in the group of patients were compared to the respective frequencies of the control group. Statistical analysis for comparisons between categorical variables (such as ethnicity, sex, age, family history of cancer or thrombophilia) was performed using the Chi-square test and Fisher's exact test. All statistical differences were two-sided, and the significance level was set at  $p < 0.05$ .

## Results

No significant differences between Greek and German subjects were observed, therefore data from both populations were analyzed together (Tables I and II). In the control group (N=120), the Leiden mutation was detected in 6 heterozygotes (5%), while the G20210A mutation was detected in 5 heterozygotes (4.2%). Therefore, the observed allele frequencies were 2.5% and 2.0% for the Leiden and the G20210A mutations, respectively. These findings are similar to those previously reported for the Greek and German populations (15, 25).

In the patient group (N=102), the Leiden mutation was detected in 5 heterozygotes (4.9%), resulting in a frequency of the mutant allele of 2.5%. The G20210A mutation was detected in 4 heterozygotes (3.9%), therefore the frequency of the mutant allele was 2.0%. For both mutations, the observed carrier and mutant frequencies of the patients compared to the equivalent ones of the controls indicated no statistical difference. Comparisons between subgroups using parameters such as positive family history for cancer, ethnicity, sex or age failed to detect any statistically significant differences in allele frequencies either. The only statistical difference with controls was observed for the Leiden allele and carrier frequencies in the subgroup of patients with a positive history of thrombophilia ( $p < 0.001$ , Table I).

## Discussion

Patients with malignancies have an increased risk of coagulopathy due to the release of tissue factor by the tumor, damage to the vessel wall and immobilization (26-28). Moreover, tumors may improve their growth and metastatic spread by utilizing the angiogenesis process (29, 30). It is

Table I. Prevalence of factor V Leiden mutation in 120 healthy controls and 102 patients with oral cancer. N.S.: no statistical difference.

	Controls	Patients	Patients with positive history of cancer	Patients without positive history of cancer	Patients with positive history of thrombophilia	Patients without positive history of thrombophilia
Heterozygotes	6	5	1	4	4	1
Homozygotes mutant	0	0	0	0	0	0
Homozygotes normal	114	97	45	52	28	69
Total	120	102	46	56	32	70
Statistical difference		N.S.	N.S.	N.S.	$p < 0.001$	N.S.
Carrier frequency	5.0%	4.9%	2.2%	7.1%	12.5%	1.4%
Allele frequency	2.5%	2.5%	1.1%	3.6%	6.3%	0.7%
Statistical difference		N.S.	N.S.	N.S.	$p < 0.001$	N.S.

Table II. Prevalence of prothrombin G20210A mutation in 120 healthy controls and 102 patients with oral cancer. N.S.: no statistical difference.

	Controls	Patients	Patients with positive history of cancer	Patients without positive history of cancer	Patients with positive history of thrombophilia	Patients without positive history of thrombophilia
Heterozygotes	5	4	1	3	2	3
Homozygotes mutant	0	0	0	0	0	0
Homozygotes normal	115	98	45	53	30	67
Total	120	102	46	56	32	70
Statistical difference		N.S.	N.S.	N.S.	N.S.	N.S.
Carrier frequency	4.2%	4.0%	2.2%	5.4%	6.3%	4.3%
Allele frequency	2.0%	2.0%	1.1%	2.5%	3.1%	2.1%
Statistical difference		N.S.	N.S.	N.S.	N.S.	N.S.

thus clear that coagulopathy and angiogenesis are among the most consistent host responses associated with cancer (3). These two processes may, in fact, be functionally inseparable as blood coagulation and fibrinolysis, in their own right, influence tumor angiogenesis and thereby contribute to malignant growth (3). In addition, tumor angiogenesis appears to be controlled through both standard and non-standard functions of such elements of the hemostatic system as tissue factor, thrombin, fibrin, plasminogen activators, plasminogen and platelets (3). "Cryptic" domains can be

released from hemostatic proteins through proteolytic cleavage, and act systemically as angiogenesis inhibitors (*e.g.*, angiostatin, antiangiogenic antithrombin III aaATIII) (3). Various components of the hemostatic system either promote or inhibit angiogenesis, probably acting by changing the net angiogenic balance (3). However, their complex influences are far from being fully understood (3). Therefore, in view of an association between the coagulation-related MTHFR mutation and oral cancer, shown in some reports (8, 10), we searched for a possible

contribution of the other two most common thrombophilia-predisposing genetic factors in oral cancer risk (15, 22-25).

The study of the factor V and prothrombin mutations was effected in 102 unrelated individuals who presented with oral cancer in comparison to 120 healthy controls of comparable ethnicity, age and sex. The overall data obtained did not reveal any association between Leiden and G20210A mutations and an increased risk for oral cancer, in contrast with the association previously reported for other types of cancer (4, 7, 17-20). The only exception was the significant findings for Leiden in the subgroup with a positive history for thrombophilia, but firm conclusions cannot be drawn because of the small number of patients studied.

Factor V and prothrombin are not the only factors in which defects predispose to cardiovascular disease. There are other genetic defects established as risk factors for venous thrombosis, such as protein C, protein S and antithrombin deficiencies, defects in the anticoagulant pathways of blood coagulation *etc.* (31). Therefore, an investigation of the possible association of other thrombophilia-related factors to increased risk of oral cancer should be conducted.

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### References

- Prime SS, Thakker NS, Pring M, Guest PG and Paterson IC: A review of inherited cancer syndromes and their relevance to oral squamous cell carcinoma. *Oral Oncol* 37: 1-16, 2001.
- Regezi JA and Jordan RC: Oral cancer in the molecular age. *J Calif Dent Assoc* 29: 578-584, 2001.
- Trikha M and Nakada MT: Platelets and cancer: implications for antiangiogenic therapy. *Semin Thromb Hemost* 28: 39-44, 2001.
- Nowak-Gottl U, Wermes C, Junker R, Koch HG, Schobess R, Fleischhack G, Schwabe D and Ehrenforth S: Prospective evaluation of the thrombotic risk in children with acute lymphoblastic leukemia carrying the MTHFR TT 677 genotype, the prothrombin G20210A variant, and further prothrombotic risk factors. *Blood* 93: 1595-1599, 1999.
- Jeng YL, Wu MH, Huang HB, Lin WY, You SL, Chu TY, Chen CJ and Sun CA: The methylenetetrahydrofolate reductase 677C-->T polymorphism and lung cancer risk in a Chinese population. *Anticancer Res* 23: 5149-5152, 2003.
- Bailey LB: Folate, methyl-related nutrients, alcohol, and the MTHFR C677T polymorphism affect cancer risk: intake recommendations. *J Nutr* 133: 3748-3753, 2003.
- Ramacciotti E, Wolosker N, Puech-Leao P, Zeratti EA, Gusson PR, del Giglio A and Franco RF: Prevalence of factor V Leiden, FII G20210A, FXIII Val34Leu and MTHFR C677T polymorphisms in cancer patients with and without venous thrombosis. *Thromb Res* 109: 171-174, 2003.
- Song C, Xing D, Tan W, Wei Q and Lin D: Methylenetetrahydrofolate reductase polymorphisms increase risk of esophageal squamous cell carcinoma in a Chinese population. *Cancer Res* 61: 3272-3275, 2001.
- Weinstein SJ, Gridley G, Harty LC, Diehl SR, Brown LM, Winn DM, Bravo-Otero E and Hayes RB: Folate intake, serum homocysteine and methylenetetrahydrofolate reductase (MTHFR) C677T genotype are not associated with oral cancer risk in Puerto Rico. *J Nutr* 132: 762-767, 2002.
- Vairaktaris E, Yapijakis C, Wiltfang J, Ries J, Vylliotis A, Vasiliou S and Neukam FW: Methylenetetrahydrofolate reductase polymorphism and increased risk of oral cancer. *Eur J Surg Oncol* (in press).
- Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA, van den Heuvel LP *et al*: A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 10: 111-113, 1995.
- Isotalo PA, Wells GA and Donnelly JG: Neonatal and fetal methylenetetrahydrofolate reductase genetic polymorphisms: an examination of C677T and A1298C mutations. *Am J Hum Genet* 67: 986-90, 2000.
- Meglic L, Stegnar M, Milanez T, Bozic M, Peterlin B, Peternel P and Novak-Antolic Z: Factor V Leiden, prothrombin 20210G-A, methylenetetrahydrofolate reductase 677C-T and plasminogen activator inhibitor 4G/5G polymorphism in women with pregnancy-related venous thromboembolism. *Eur J Obstet Gynecol Reprod Biol* 111: 157-163, 2003.
- Berrut G, Ghali A, Quere I, Ternisien C, Gallois I, Roy PM, Marre M and Fressinaud P: A common mutation C677T in the 5,10-methylenetetrahydrofolate reductase gene is associated to idiopathic deep venous thrombosis. *Rev Med Interne* 24: 569-576, 2003.
- Antoniadi T, Hatzis T, Kroupis C, Economou-Petersen E and Petersen MB: Prevalence of factor V Leiden, prothrombin G20210A, and MTHFR C677T mutations in a Greek population of blood donors. *Am J Hematol* 61: 265-267, 1999.
- Dowling NF, Austin H, Dilley A, Whitsett C, Evatt BL and Hooper WC: The epidemiology of venous thromboembolism in Caucasians and African-Americans: the GATE study. *J Thromb Haemost* 1: 80-87, 2003.
- Zacharski LR, Memoli VA, Rousseau SM and Kisiel W: Occurrence of blood coagulation factors *in situ* in small cell carcinoma of the lung. *Cancer* 60: 2675-2681, 1987.
- Pihusch R, Danzl G, Scholz M, Harich D, Pihusch M, Lohse P and Hiller E: Impact of thrombophilic gene mutations on thrombosis risk in patients with gastrointestinal carcinoma. *Cancer* 94: 3120-3126, 2002.
- Kyriakou DS, Alexandrakis MG, Passam FH, Foundouli K, Matalliotakis E, Koutroubakis IE, Kourelis TV, Chalkiadakis G and Manoussos ON: Acquired inhibitors to coagulation factors in patients with gastrointestinal diseases. *Eur J Gastroenterol Hepatol* 14: 1383-1387, 2002.
- Kim RJ and Becker RC: Association between factor V Leiden, prothrombin G20210A, and methylenetetrahydrofolate reductase C677T mutations and events of the arterial circulatory system: a meta-analysis of published studies. *Am Heart J* 146: 948-957, 2003.

- 21 Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, van der Velden PA and Reitsma PH: Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 369: 64-67, 1994.
- 22 Bertina RM: Factor V Leiden and other coagulation factor mutations affecting thrombotic risk. *Clin Chem* 43: 1678-1683, 1997.
- 23 Ridker PM, Hennekens CH and Miletich JP: A G20210A mutation in prothrombin gene and risk of myocardial infarction, stroke, and venous thrombosis in a large cohort of US men. *Circulation* 99: 999-1004, 1999.
- 24 Poort SR, Rosendaal FR, Reitsma PH and Bertina RM: A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood* 88: 3698-3703, 1996.
- 25 Pauer HU, Voigt-Tschirschwitz T, Hinney B, Burfeind P, Wolf C, Emons G and Neesen J: Analyses of three common thrombophilic gene mutations in German women with recurrent abortions. *Acta Obstet Gynecol SCAND* 82: 942-947, 2003.
- 26 Lee AY and Levine MN: Venous thromboembolism and cancer: risks and outcomes. *Circulation* 107: 117-121, 2003.
- 27 Prandoni P: Cancer and thromboembolic disease: how important is the risk of thrombosis? *Cancer Treat Rev* 32: 133-136, 2002.
- 28 Mousa SA: Anticoagulants in thrombosis and cancer: the missing link. *Expert Rev Anticancer Ther* 2: 227-233, 2002.
- 29 Nash GF, Walsh DC and Kakkar AK: The role of the coagulation system in tumor angiogenesis. *Lancet Oncol* 2: 608-613, 2001.
- 30 Wojtukiewicz MZ, Sierko E, Klement P and Rak J: The hemostatic system and angiogenesis in malignancy. *Neoplasia* 3: 371-384, 2001.
- 31 Murin S, Marelich GP, Arroliga AC and Matthay RA: Hereditary thrombophilia and venous thromboembolism. *AM J Respir Crit Care Med* 158: 1369-1373, 1998.

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