ORIGINAL RESEARCH

Role of MMP9 and TIMP2 Gene Polymorphisms in Varicose Veins Formation in the Population of North India

[1]Sushil Kumar Bundela, [2]Pushpendra Agarwal, [3]Mohit Jain, [4]Ramesh Yadav

[1]Assistant Professor, Department of General Surgery, Government Medical College, Jalaun (Orai), UP, India
[2]Associate Professor, Department of General Surgery, Government Medical College, Jalaun (Orai), UP, India
[3]Assistant Professor, Department of General Surgery, Autonomous State Medical College, Lalitpur, UP, India
[4]Associate Professor, Department of Microbiology, Government Medical College, Jalaun (Orai), UP, India

Corresponding Author
Ramesh Yadav
Associate Professor, Department of Microbiology, Government Medical College, Jalaun (Orai), UP, India
Email: microramesh001@gmail.com

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ABSTRACT

Aim and Objective: To find out Role of MMP9 and TIMP2 gene polymorphisms in the varicose veins formation in North Indian population. **Methods:** A total of 600 (300 cases and 300 controls) individuals were selected for the study, who had diagnosed for varicose veins complication from January 2020 to July 2020 at Government Medical College, Jalaun (Orai) UP. (India). Cases and controls age was between 10-60 years. Fresh 5 ml of EDTA blood was drawn for genetic analyses from both patients and controls as per standardized protocols. For the identification of MMP-9 and TIMP-2 genes polymorphism, DNA were isolated from the fresh blood by using Qaigen kit protocol. PCR-RFLP was performed to determine genotype variants. **Results:** The 50% males and 50% females were selected for this study. 59 (19.66%) cases were reported those had family history of varicose veins formation while it was found quite less 8 (0.02%) in the controls with OR=44.22, 95% Cl=14.78, p-value <0.0001. In the PCR-RFLP **t**he distribution of genotypes frequencies in the cases and controls groups it has found that gene frequencies of MMP-9 genotypes have not found statistical significance, whereas the gene frequency of TIMP-2, GG (homozygous) genotype was found statistically significant which was higher in the controls than in the cases. **Conclusion:** To our knowledge, this is the first study to report on MMP-9 and TIMP-2 gene polymorphisms in relation to varicose veins formation in north Indian population. In our sense, such type of studies are required to know the strength and exact nature of the genetic variation to know which of the variant within a haplotype cluster could be functionally related to varicose veins formation in the populations. This study may have demonstrated a statistically significant result in the studied population in large group.

Key Words: Varicose vein formation, Matrix Metallo-proteinase-9, Tissue Inhibitor of Matrix Metallo-proteinase-2, Gene polymorphism, Genotypes

INTRODUCTION

Development of varicose veins and persistent venous shortage are the most common disorders of the lower limbs. It is more common in females than males of human being.[1] There are several factors are associated for this disease such as old age, ligamentous laxity, prolonged standing, pregnancy, obesity, leg trauma and venous thromboses.[2-7] There are few more risk factors involved to cause this disease such as family history and genetic abnormalities for varicose vein formation. The risk of developing varicose veins has been proven for an individual is around 90% when parents suffered from varicose disease. The probability of this disease is around 20% when parents are normal.[8] The inner wall of blood vein is known as tunica intima which is made up of smooth endothelial lining surrounded by an extracellular matrix (ECM) consisting of elastin, collagen and proteoglycans. The elastin and collagen are important structures and responsible for maintaining the integrity of vein wall. This layer has also contains valves to keep the blood flowing in a single upward direction towards the heart.[9,10] There are four mechanisms are known which may be accountable for the patho-physiology of varicose vein formation. First one is due to loss of elasticity of the vein wall, mainly due to increasing age. The second one is due to absence or non functioning of the venous valves. In the third case it may be due to dysfunctioning of muscular pumping system. In the fourth cases it may

be due to the difficulties of the venous system. All these obstructions may cause venous hypertension and dilated veins in the lower extremities.[10,11] Matrix Metallo-proteinases (MMPs) is a group of more than 24 endopeptidases (enzymes). All the enzymes are neutral and catalyze the degradation of the proteins in the ECM. The degradation of proteins in ECM is a well in order and performs many physiological functions such as angiogenesis, tissue remodeling and wound healing. However, due to over-expression of MMP gene several mul-functioning may be arises *e.g.* varicose veins formation, cancer, autoimmune diseases, cardiovascular diseases etc.[12] One of the most important Matrix metalloproteinases-9 (MMP9) is a member of the gelatinase family called gelatinase B which is responsible to degrades collagen types IV, V, and XI, telopeptides of type I collagen along with elastin.[13] In begin, MMP9 is produced in inactive zymogenic form and needs enzymatic activation. MMP activity is regulated by α_2-macroglobulin and Tissue Inhibitors of Matrix Metallo-proteinases (TIMPs).[14,15] TIMPs are responsible for the control of the activation of the proenzyme and substrate degradation.[16] The TIMP family has four members synthesized in vascular smooth muscle cells, endothelial cells and macrophages.[17] The expression of MMP1, 2, 3, 9 and 13 is increased in patients of varicose veins formation. The key reason of this increase is may be due to an imbalance between the activities of MMPs and TIMPs, by which in ECM accumulated in the vein wall.[18,19] The genetic polymorphisms of MMPs and TIMPs are may be important determining factors for this imbalance. The aim of this study was to identify and evaluate the genetic polymorphisms of MMP9 and TIMP2 genes in order to varicose vein formation and examine these polymorphisms. The study also focuses to relate MMP-9 and TIMP-2 gene polymorphism with the relation of varicose vein formation of North Indian population.

MATERIALS AND METHODS

This study was performed at Government Medical College, Jalaun (Orai) UP. (India). This medical college has a tertiary health care association. All the reagents and chemicals were ordered from different suppliers such as Sigma Aldrich, Fermentas, Bio-Rad and Qaigen. For the study total 600 samples (300 cases and 300 controls) were considered. Before the collection of samples a written signed consent was taken from all the cases and controls along with a filled questionnaire form. This study was performed between January 2020 to July 2020 and entire protocol was approval by the ethics committee of the Government Medical College, Jalaun (Orai) UP. (India). Cases and controls age was between 10-60 years. Family history record of cases and controls was also collected. The patients were categorized based on clinical, etiological, anatomical and pathophysiological.[20] High ligation with stripping with or without additional individual ligation and excision were performed for symptoms, complications or cosmetic needs. 2.0 ml of EDTA blood was collected for genetic analyses from each sample.

Sample Size: Sample size was calculated by using below mentioned formula wherein the calculated minimum sample size was found for our study is 150.[21]

$$N = \frac{(Z_{1-\alpha/2+Z1-\beta})\,(\sigma_1^2 + \sigma_2^2)}{(\mu_1^2 - \mu_2^2)}$$

μ_1=64.27 μ_2= 70.615

σ_1= 13.505 σ_2= 16.36

$Z_{1-\alpha 2}$ =1.96 for 95% confidence, $Z_{1-\beta}$ = 1.84 for 80% power

Inclusion Criteria: Normal persons above the age of 10 years were recruited as controls. The persons suffering from varicose veins disease with no confounding factors will be recruited as subjects for the study. Individual who needed ligation and excision symptoms were also included as subjects for this study.

Exclusion Criteria: The following exclusion criteria's were needed for the cases and controls as mentioned below.

1. Individuals who had previous history of diabetes mellitus.
2. Individuals known risk of thyroid disease e.g. hypothyroidism or hyperthyroidism and taking thyroid medication.
3. Individuals with hormonal replacement therapy, taking vitamin D supplements.
4. Individuals with viral infections, tuberculosis, cancer, mental illness, epilepsy and acute inflammation.

Biochemical Measurement: Control persons were selected in the hospital according standard protocol of WHO *i.e.* fasting plasma glucose <7.0 mmol/l, and history of non management of hypoglycemic record. High ligation with stripping with or without additional individual ligation and excision were performed for symptoms, complications or cosmetic needs.[22] The varicose veins disease was confirmed and examined by senior surgeons of surgery department. There were several clinical examinations tested *e.g.* edema and skin trophic modifications on the legs as regular examination. This disease was also confirmed by the department of radiology with the use of duplex ultrasonography scan. A questionnaire was collected from patients who were related to venous complications *e.g.* skin trophic modifications, family history of thromboembolic disease, history of varicose veins, exercise activities and occupations etc. Fresh 5 ml of EDTA blood was drawn for genetic analyses from both patients and controls as per standardized protocols. For the identification of MMP-9 and

TIMP-2 genes polymorphism, first DNA were isolated from the fresh collected blood by using Qaigen kit with following user manual. Purity of DNA was analyzed by running 1% agarose gel and by estimating the 260/280 nm absorbance. Primers were synthesized from Chromous Biotech Pvt. Ltd, Bangaluru. Isolated DNA was amplified with the use of PCR (Bio-RAD, T-100). The PCR conditions were as; initial denaturation for 5 min at 95˚C, for 35 cycles, denaturation at 94˚C for 30 sec, 56˚C for 30 sec, 72˚C for 1 min and for final extension at 72˚C for 7 min for MMP-9 gene. The only difference in the annealing temperature (55˚C for 30 seconds) for the amplification of TIMP-2 gene and rest conditions were similar as MMP-9gene. Amplified DNA was resolved with 1.0% agarose gel containing ethidium bromide and bromophenol blue. Gel photographs were analyzed with software connected in gel documentation system (Bio-RAD).[23] For the analysis of restriction fragment length polymorphism (RFLP) of MMP-9 and TIMP-2 genes PAE-I and HGA-I restriction endonuclease enzymes (Fermentas Pvt. Ltd.) were used respectively with following standard digestion protocol.[24] Digested fragments were electrophoresis in a 2% agarose gel for allele identification. The statistical analyze was performed by using SPSS software version 20 (USA). Statistical power of the study was analyzed under log-additive model, assuming 10% population possibility by Quanto. Means and standard deviations values were calculated by inverse normal units of the parameters. Allele frequencies of cases and controls of the study population were analyzed by equal opportunity of proportions Z-test. The odds ratio (OR) and 95% confidence interval (CI) were calculated with respect to minor alleles.[25]

Table 1: Primers oligoes for the Amplification of MMP-9 and TIMP-2 Genes

S.N.	Primer sequences	Tm (ºC)
1.	*MMP-9* F (5'- GCCTGGCACATAGTAGGCCC-3')	59.0
2.	*MMP-9* R (5'-CTTCCTAGCCAGCCGGCATC-3')	60.0
3.	*TIMP-2* F (5'- GATCCTGTCAGTTTCTCAA-3')	58.5
4.	*TIMP-2* R (5'-TTTCCCCTTAGCTCGACTCT-3')	61.0

RESULTS

Clinical and Biochemical Measurement

The study was conducted with the 300 cases and 300 controls of randomly selected varicose veins patients and healthy persons. Demographic data of the patients and controls are mentioned in Table 2. Assessments of age, BMI, systolic and diastolic blood pressures and smoking have showed no statistically significant differences between the groups. The BMI was estimated according to the WHO Asia pacific guidelines as non-obese (BMI <25 kg/m^2) and obese (BMI >25 kg/m^2). Though, evaluations of gender and family history record have demonstrated a significant difference between groups with significant P value <0.0001. The most of the data analyzed values were shown significantly greater in cases with respect to controls. The cases and controls were 150 (50%) males and 150 (50%). The family history of varicose veins were found positive in 59 (19.66%) cases, whereas it was found positive only in 8 (0.02%) in the controls with OR=44.22, 95% Cl=14.78, p-value <0.0001). In the Fig. 1, isolated DNA from the cases and controls are presented. In the Fig. 2 and 3 demonstrated the data of PCR-RFLP of the 1562C/T polymorphism of MMP-9 and 418G/C polymorphism of TIMP-2 gene. The genotypic frequency distributions of MMP-9 and TIMP-2 had not illustrated any significant deviation from the predictable Hardy-Weinberg equilibrium in the any studied groups. Table 3 showed the distribution of allelic genotypic frequencies in the cases and controls groups. Frequencies of MMP-9 genotypes have not found statistical significance results between patients and controls, whereas the frequency of TIMP-2, GG (homozygous) genotype was found statistically significant higher in the controls than in the cases.

Table 2: **Demographic Data of the Studied Cases and Controls**

S.N.	Clinical Characteristics	Cases	Controls	*P*-value
1.	No. of person involved	300	300	-
2.	No. of male gender	150	150	-
3.	Mean age ±SD (years)	52.36	54.18	0.001
4.	BMI (Kg/m2) ±SD	24.13±1.4	22.42±1.4	0.110
5.	No. of Smoking persons	102	95	0.001
6.	Systolic BP (mmHg) ±SD	120±1.5	119±1.4	<0.005
7.	Diastolic BP (mmHg) ±SD	80±4.0	80±1.7	<0.005
8.	Positive family record	59	08	<0.0001

Table 3: **Genotypes frequencies distribution of MMP-9 and TIMP-2 alleles in patients of varicose veins and the control group**

Gene	Genotype	Cases (n=300), n (%)	Controls (n=300), n (%)	Adjusted OR	95% CI	*p*-value
MMP-9 genotype	CC	0.00	0.00	-	-	-
	CT	108 (36%)	60 (20%)	1.326	0.74-1.27	0.756
	TT	192 (64%)	240 (80%)	0.945	0.83-1.13	0.367
TIMP-2 genotype	CC	0.00	0.00	-	-	-
	CG	252 (84%)	196 (64%)	1.201	0.75-1.81	0.401
	GG	48 (16%)	104 (36%)	0.391	0.18-0.84	0.010

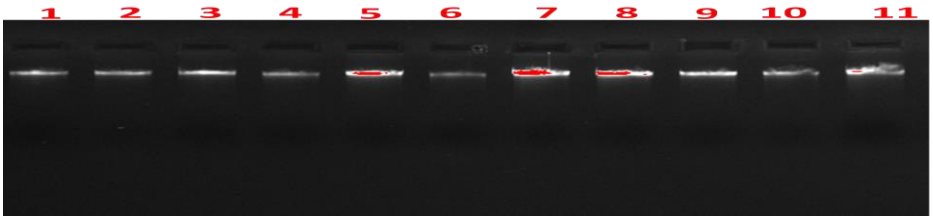

Figure 1: Photograph of Isolated DNA (MMP 9 and TIMP2 gene)

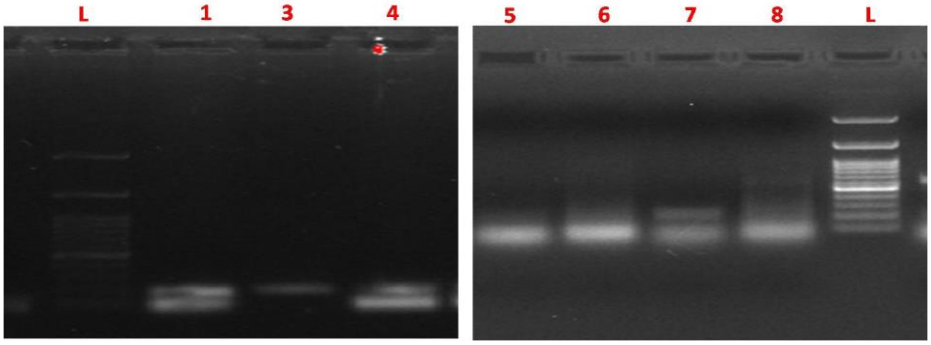

Figure 2 and 3: PCR-RFLP analysis of 1562C/T polymorphism of MMP9 promoter and 418G/C polymorphism of TIMP2 gene promoters. Lane 1-3 corresponded to MMP 9 gene while lane 5-8 corresponded to the TIMP gene, L represented 100bp ladder

DISCUSSION

In the available literature there are few genetic studies have been mentioned about the MMP-9 and TIMP-2 gene polymorphisms in relation to varicose veins formation. But detailed study with genotypes variation for this region is not studied so far. Varicose veins formation on lower extremity is mainly a cosmetic problem due to poor quality of life as a result of chronic venous shortage. In the present study, genotypes of MMP-9 have not statistically significantly in the patients and controls. MMPs are the main proteinases which humiliating the extracellular matrix.[26] MMP-9 plays a crucial role in the digestion of denatured collagens. It has mentioned in the literature that MMPs have been played a big role in many tissues including cardiovascular system. The MMP expression had been increased mechanical pressure in tissues and responsible in varicose veins formation due to amplified venous hydrostatic pressure[27] resulting increased hydrostatic pressure in the veins and cell permeability, primarily to leucocyte infiltration, smooth muscle proliferation and finally involved in venous valvular degeneration.[28] It has been reported that polymorphisms in the promoter region of MMP-9 is related to the varicose veins in the Chinese population.[29] In the present study identical results have been obtained in relation between polymorphism of MMP-9 and varicose veins formation. There are similar finding had been obtained in Turkish study population.[30] The Chinese study explained that those with CC genotype had a greater risk of varicose veins formation, though, in this study none of the cases and controls were carriers the CC genotype. This may be due to the race differences.[31] TIMPs are small proteins and regulate the action of MMPs and inhibit MMPs activities by binding to their active zinc-binding area.[32] In this study, CG (heterozygous) genotypes is responsible for higher risk of varicose veins formation while individuals with the GG genotype may have a lower risk for varicose vein formation. The anti-angiogenic properties of the endogenous TIMPs are well recorded. TIMP-2 plays a key role in the inhibition of endothelial cell proliferation.[33]

CONCLUSION

To our knowledge, this is the first study to report on MMP-9 and TIMP-2 gene polymorphisms in relation to varicose veins formation in north Indian population. In our sense, such type of studies are

required to know the strength and exact nature of the genetic variation to know which of the variant within a haplotype cluster could be functionally related to varicose veins formation in the populations. This study may have demonstrated a statistically significant result in the studied population in large group.

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DECLARATIONS

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Conflicts of interest: The authors are thankful to the Government Medical College, Jalaun (Orai) UP (India) for the financial support.

Ethics approval: This study was approved by the ethical committee of Government Medical College, Jalaun (Orai) UP (India).

Consent to participate: We have consent to participate.

Consent for publication: We have consent for the publication of this paper.

Availability of data and material: The entire data and materials were self generated.

Code availability: Not applicable.

Authors' contributions: Sushil Kumar Bundela and Ramesh Kumar Yadav both the authors were equally contributed to the work at Government Medical College, Jalaun (Orai) UP (India).

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REFERENCES

1. Cauwe B, Van den Steen PE, Opdenakker G (2007): The biochemical, biological, and pathological kaleidoscope of cell surface substrates processed by matrix metalloproteinases. Crit Rev Biochem Mol Biol 42: 113-185
2. Beebe-Dimmer JL, Pfeifer JR, Engle JS and Schottenfeld D: The epidemiology of chronic venous insufficiency and varicose veins. Ann Epidemiol 15: 175-184, 2005.
3. 2 Baker SR, Stacey MC, Jopp-McKay AG, Hoskin SE and Thompson PJ: Epidemiology of chronic venous ulcers. Br J Surg 78: 864-867, 1991.
4. Criqui MH, Denenberg JO, Bergan J, Langer RD and Fronek A: Risk factors for chronic venous disease: the San Diego Population Study. J Vasc Surg 46: 331-337, 2007.
5. Scott TE, LaMorte WW, Gorin DR and Menzoian JO: Risk factors for chronic venous insufficiency: a dual case-control study. J Vasc Surg 22: 622-628, 1995.
6. Chiesa R, Marone EM, Limoni C, Volonte M and Petrini O: Chronic venous disorders: correlation between visible signs, symptoms, and presence of functional disease. J Vasc Surg 46: 322-330, 2007.
7. Stacey MC, Burnand KG, Lea Thomas M and Pattison M: Influence of phlebographic abnormalities on the natural history of venous ulceration. Br J Surg 78: 868-871, 1991.
8. Hanrahan LM, Araki CT, Rodriguez AA, Kechejian GJ, LaMorte WW and Menzoian JO: Distribution of valvular incompetence in patients with venous stasis ulceration. J Vasc Surg 13: 805811, 1991.
9. Cornu-Thenard A, Boivin P, Baud JM, De Vincenzi I and Carpentier PH: Importance of the familial factor in varicose disease. Clinical study of 134 families. J Dermatol Surg Oncol 20: 318-326, 1994.
10. Naoum JJ, Hunter GC, Woodside KJ and Chen C: Current advances in the pathogenesis of varicose veins. J Surg Res 141: 311-316, 2007.
11. Vairaktaris E, Serefoglou Z, Yapijakis C, Vylliotis A, Nkenke A, et al. (2007): High gene expression of matrix metalloproteinase-7 is associated with early stages of oral cancer. Anticancer Res 27: 2493-2498.
12. London NJM, Nash R: ABC of arterial and venous disease. Varicose veins. BMJ 320: 1391-1394, 2000.
13. Hinden E: Valvular defect in varicose veins Lancet1: 491-492, 1964.
14. Venturi M, Bonavina L and Annoni F: Biochemical assay of collagen and elastin in the normal and varicose vein wall. J Surg Res 60: 245-248, 1996.
15. Yan C and Boyd DD: Regulation of matrix metalloproteinase gene expression. J Cell Physiol 21: 19-26, 2007.
16. Visse R and Nagase H: Matrix metallo-proteinases and tissue inhibitors of metallo-proteinases. Circ Res 92: 827-839, 2003.
17. Murphy G: The regulation of connective tissue metallo-proteinases by natural inhibitors. Agents Actions Suppl 35: 6976, 1991.
18. Nagase H, Visse R and Murphy G: Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res 69: 562573, 2006.
19. Nagase H and Woessner JF: Matrix metalloproteinases. J Biol Chem 274: 21491-21494, 1999.
20. Gillespie DL, Patel A and Fileta B: Varicose veins possess greater quantities of MMP1 than normal veins and demonstrate regional variation in MMP1 and MMP13. J Surg Res 106: 233238, 2002.
21. Kowalewski R, Sobolewski K, Wolanska M and Gacko M: Matrix metalloproteinases in the vein wall. Int Angiol 23: 164169, 2004.
22. Porter JM and Moneta GL: Reporting standarts in venous disease: an update. International Concensus Committee on Chronic Venous Disease. J Vasc Surg 21: 635-645, 1995.
23. Miller SA, Dykes DD and Polesky HF: A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16: 1215, 1988.
24. Raffetto JD, Qiao X, Koledova VV and Khalil RA: Prolonged increases in vein wall tension increase matrix metalloproteinases and decrease constriction in rat vena cava: Potential implications in varicose veins. J Vasc Surg 48: 447-456, 2008.
25. Schmid-Schonbein GW, Takase S and Bergan JJ: New advances in the understanding of the pathophysiology of chronic venous insufficiency. Angiology 52: S27-34, 2001.
26. Raffetto JD, Ross RL and Khalil RA: Matrix metalloproteinase 2-induced venous dilatation via hyperpolarization and activation of K+ channels:

elevance to varicose vein formation. J Vasc Surg 45: 373-380, 2007.
27. Xu HM, Zhao Y, Zhang XM, Zhu T and Fu WG: Polymorphisms in MMP9 and TIMP2 in Chinese patients with varicose veins. J Surg Res 168: 143-148, 2011.
28. Stamenkovic I: Matrix metalloproteinases in tumor invasion and metastasis. Semin Cancer Biol 10: 415-433, 2000.
29. Johnson JL, Baker AH and Oka K: Suppression of atherosclerotic plaque progression and instability by tissue inhibitor of metalloproteinase-2: involvement of macrophage migration and apoptosis. Circulation 113: 2435-2444, 2006.
30. Yang L, Gu HJ, Zhu HJ, Sun QM, Cong RH, Zhou B, Tang NP and Wang B: Tissue inhibitor of metalloproteinase-2 G-418C polymorphism is associated with an increased risk of gastric cancer in a Chinese population. Eur J Surg Oncol 34: 636-641, 2008.
31. Lin TH, Chiu HC, Lee YT, Su HM, Juo SH, Voon WC, Lai WT and Sheu SH: The C-allele of tissue inhibitor of metallo-proteinases 2 is associated with increased magnitude of QT dispersion prolongation in elderly Chinese-4-year follow-up study. Clin Chim Acta 386: 87-93, 2007.
32. Chen D, Wang Q and Ma ZW: MMP2, MMP9and TIMP2gene polymorphisms in Chinese patients with generalized aggressive periodontitis. J Clin Periodontol 34: 384-389, 2007.
33. Hirano K, Sakamoto T and Uchida Y: Tissue inhibitor of metallo-proteinases-2 gene polymorphisms in chronic obstructive pulmonary disease. Eur Respir J 18: 748-752, 2001.