Grażyna Szumczyk-Muszytowska[1], Jacek Manitius[2], Andrzej Tretyn[3], Joanna Jarkiewicz-Tretyn[4], Marek Muszytowski[5]

PRACA ORYGINALNA

[1]Cardiology Department, Outpatient Clinic Lecznice Citomed, Toruń
[2]Department of Nephrology, Hypertension, and Internal Disease, Collegium Medicum, Nicolaus Copernicus University, Bydgoszcz
[3]Department of Biotechnology, Institute of General and Molecular Biology, Nicolaus Copernicus University, Toruń
[4]Cancer Genetics Laboratory, Toruń
[5]Department of Nephrology and Internal Medicine, Ludwik Rydygier Hospital, Toruń

The association between 3020INSC mutation in *NOD2* gene and arterial stiffness in hypertensives

Summary

Background The innate immune system elicits an inflammatory process which is believed to be involved in the development of vascular damage in hypertension.

The *NOD2* gene (CARD15) is involved in the control of the innate immune system. The aim of this study was to assess the occurrence of the 3020insC mutation in the *NOD2* gene in relation to vascular damage in hypertensives.

Materials and methods In 466 hypertensives, genotyping for 3020insC mutation, cholesterol, serum creatinine, and echocardiography were carried out. Systolic (SBP) and diastolic (DBP) blood pressure and pulse pressure (PP) were taken at 2–3 month intervals for one year and treated according to ESC/ESH guidelines.

Results The 3020insC mutation was found in 7.08% of hypertensives and was associated with earlier onset of hypertension 48.2 ± 12.1 vs. 42.8 ± 10.9, $p < 0.01$ (age of pts). After one year of treatment, patients with the mutation had significantly higher SBP (138.48 ± 18.35 vs. 131.74 ± 14.97, $p < 0.04$) and PP (57.27 ± 14.04 vs. 52.70 ± 12.96, $p < 0.04$) than patients without the mutation, whereas the number of drugs was the same in both groups.

Conclusions The results suggest that the 3020insC mutation is associated with earlier onset of hypertension and increased arterial stiffness

key words: arterial stiffness, innate immunity, *NOD2* mutation

Arterial Hypertension 2009, vol. 13, no 6, pages 371–375.

Adres do korespondencji: prof. dr hab. n. med. Jacek Manitius
Department of Nephrology, Hypertension, and Internal Medicine
University Hospital no. 1
Skłodowskiej-Curie 9, 85–094 Bydgoszcz
Tel/fax: (+48) 52 585 4030
e-mail: nerka@nerka.cpro.pl

Introduction

The structural and functional changes of vascular vessels characterize arterial hypertension. Remodelling of vessels results in thickening of the artery wall, increased pulse pressure, and atherosclerosis [1, 2]. Many factors are believed to be involved in the development of vascular damage in hypertension, and recently, innate and adaptive immune mechanisms are recognized as a cause of atherosclerosis and remodelling of vessels [3–6]. The innate immune system elicits inflammatory response to various pathogens via various receptors [7–9]. The *NOD2* gene (also known as CARD15), discovered in 2001, is believed to be involved in the control of the innate immune system activating nuclear factor kB and causing the inflammatory reaction and apoptosis [10–16]. Apoptosis and inflammation play crucial roles in the process of the of remodelling of arteries in hypertension [17]. Several mutations have been identified in the *NOD2* gene, which are linked to inflammation and apoptosis. One of them, frameshift mutation 3020insC (1007fs), occurs in the European population relatively often and has been proven to be associated with the inflammatory process [18–20]. In 2005 the *NOD2* gene was discovered in human endothelium [21, 22]. Recently, it was shown that allelic variants of *NOD2* were associated with preeclampsia syndrome with susceptibility to severe hypertension [23]. In the face of the relation between *NOD2* mutation and blood pressure control in preeclampsia, it appears very likely that mutation in the *NOD2* gene is associated with vascular vessel function and structure. We tested only the 1007fs mutation in the *NOD2* gene because our previous study showed that frequency *NOD2* gene variants (1007fs,G908R and R702W and P268S polymor-

phism) in the Polish population of patients with rectal cancer was found in the 1007fs mutation and there is an association between frequency of cancer and age [23–25].

The aim of the study was to see whether the occurrence of the 3020insC mutation in the *NOD2* gene is associated with the clinical manifestation of cardiovascular damage in hypertensives.

Materials and methods

The study included 466 hypertensives treated in the Cardiology Outpatient Clinic in Lecznice Citomed in Toruń. The study was in accordance with the Declaration of Helsinki and was approved by the Commission of Bioethics at Collegium Medicum of Nicolaus Copernicus University in Toruń. Informed consent was obtained from all subjects.

The enrolment examination included medical history, current therapy, previously diagnosed cardiovascular diseases, physical examination, and BMI. Fasting blood was sampled in tubes with EDTA for genotyping, total cholesterol, and creatinine and stored at –80°C. eGFR was estimated according to MDRD formula. Total cholesterol was determined enzymatically, and creatinine according to Jaffe's method. Left ventricular mass (LVM) was estimated using a Vivid 3 GE echocardiograph with a 2.5 MHz ultrasound probe according to Penn convention and Devereux formula and expressed as left ventricular mass index (LVMI) calculated as LVM/body surface area [16].

Blood pressure measurements (BP) were taken at 2–3 month intervals over one year in accordance with ESC/ESH 2003 guidelines. The mean of two consecutive measurements were reported. The target blood pressure values were < 140 mm Hg systolic (SBP) and < 90 mm Hg diastolic (DBP) for nondiabetics. Pulse pressure (PP) was calculated according to the equation PP = SDP-DBP. All patients were subjected to antihypertensive therapy according to ESC/ESH guidelines.

Genotyping

The 3020insC mutation was detected using PCR reaction [4]. For the PCR reaction, four different primers were used: "3020sens", "3020anty", "3020wild", and "3020mut", forming three different pairs. The "3020wild" and "3020mut" primers have a wild sequence and a 1007fs mutation equivalent, respectively, on their 3' ends. The thermal conditions of the PCR reaction and the concentrations of the substrates were adjusted so the highest specificity could be obtained. If the 3020insC mutation appeared, the "3020mut" primer bound to the DNA on the template while the "3020insC" primer did not. Afterwards, the product of the reaction was subjected to electrophoresis. An exemplary photogram of electrophoresis with marked 3020insC mutation is shown in figure 1.

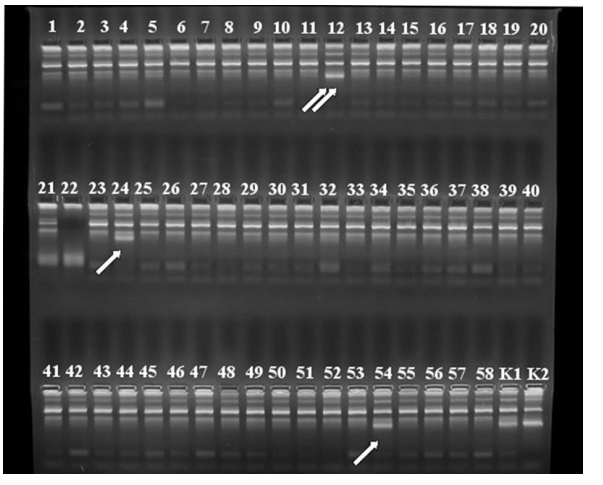

Figure 1. Electrophoretic separation of PCR reaction product. The 3020insC mutation is marked by arrows. Single arrow — heterozygote, double arrow — homozygote, K1 — control test: heterozygote, K2 — control test: homozygote

Statistical analysis

Statistical analysis was performed with SPSS 12.0 PL software and Microsoft Excel calculation sheet. The patients included in the study were divided into two groups: one group consisted of patients with identified 3020insC mutation in *NOD2* (*NOD2*(+)), and the second group comprised patients with negative results (*NOD2(–)*. For each variable, mean value and standard deviation were calculated using t-Student or Mann-Whitney test. Shapiro-Wilk test was used to estimate normality of distribution. $p < 0.05$ was considered significant. The ANCOVA test was applied to compare mean values between the groups. Linear regression analysis was used to evaluate the correlation between variables.

Results

3020insC mutation in the *NOD2*/CARD15 gene was found in 33 hypertensives (7.08%) (16 females and 17 males). The *NOD2*(+) was associated with earlier onset of hypertension (tab. I), and higher systolic blood pressure and pulse pressure in the face of the same number of taken antihypertensive drugs (tab. II, fig. 2). LVMI and eGFR mean values did

Table I. Clinical characteristics of patients in *NOD2*(–) and *NOD2*(+) groups (mean ± SD)

Parameter	*NOD2*(–) n = 433	*NOD2*(+) n = 33	p
Age (years)	59.4 ± 9.5	56.4 ± 10.4	NS
Onset of hypertension (patient age)	48.2 ± 12.1	42.8 ± 10.9	0.01
BMI [kg/m^2]	29.5 ± 4.3	30.00 ± 4.8	NS
Total cholesterol [mg/dl]	223.1 ± 47.6	216.9 ± 48.1	NS
GFR [ml/min/1.73 m^2]	86.9 ± 23.5	94.1 ± 27.1	NS
LVMI [g/m^2]	117.3 ± 24.5	124.2 ± 28.3	NS

Table II. Comparisons of systolic and diastolic blood pressure and pulse pressure among *NOD2*(–) and *NOD2*(+) (mean ± SD) patients

Blood pressure [mm Hg]	*NOD2*(–)	*NOD2*(+)	p
SBP 1	151.00 ± 22.12	149.09 ± 22.69	NS
DBP 1	90.25 ± 44.78	91.36 ± 15.68	NS
PP 1	62.39 ± 14.90	57.73 ± 13.41	NS
SBP 2	131.74 ± 14.97	138.48 ± 18.35	0.04
DBP 2	78.90 ± 7.40	81.52 ± 9.40	NS
PP 2	52.70 ± 12.96	57.27 ± 14.04	0.04

SBP — systolic blood pressure, DBP — diastolic blood pressure, PP — pulse pressure, 1 — at the beginning of observation, 2 — after one year of observation

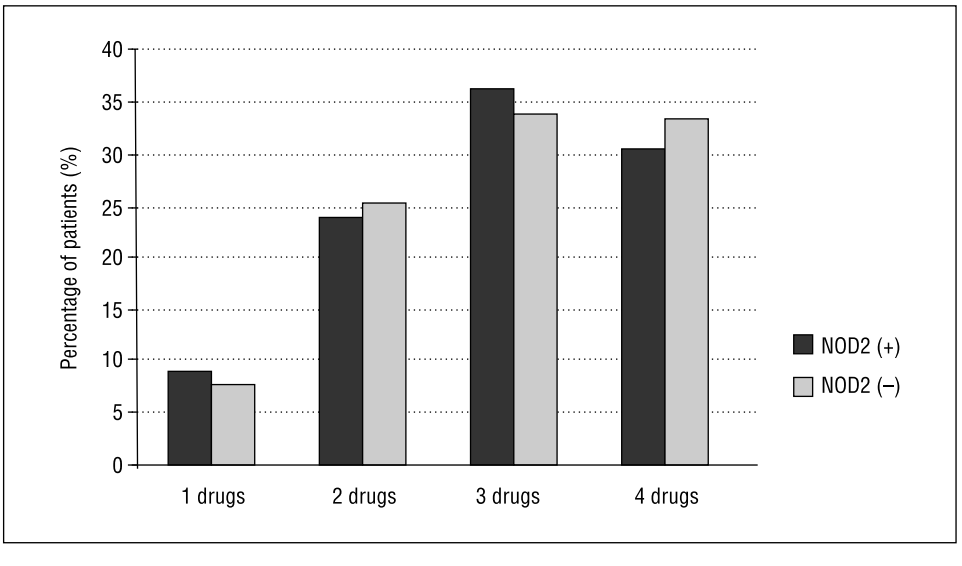

Figure 2. Number of antihypertensive drugs given to *NOD2*(+) and *NOD2*(–) patients

not differ significantly in respect to the appearance of the 3020insC mutation (tab. I). In multivariate analysis gender, SBP and PP were all predictive of LVMI ($p = 0.00$; $p = 0.03$; $p = 0.04$, respectively), while GFR was influenced only by gender ($p = 0.02$). None of these parameters appeared to be influenced by the presence of the 3020insC mutation.

Discussion

This cross-sectional study suggests that the 3020insC mutation in the *NOD2*/CARD15 gene predisposes to earlier onset of hypertension and worse blood pressure control. It is known that the frequency of these mutations is related to some inflammato-

ry diseases and cancer, but there is no available study concerning the relation between the mutation and the clinical course of hypertension [25–27]. The mechanism/s underlying the association between *NOD2* gene mutation and the pathogenesis of hypertension is/are still matter of speculation. *NOD2* has been identified as playing a role in innate immune response and consequently initiates the inflammatory process leading to activation of monocytes and macrophages. Activated cells bind to endothelium to initiate the inflammatory process in vascular vessels, which is associated with reduced artery compliance [28, 29]. Patients with the 3020insC mutation are characterized with higher systolic blood pressure and pulse pressure than subjects without mutation. This suggests that the appearance of the mutation could have been associated with more advanced inflammatory processes in the vessels, causing higher vascular stiffness. Our study also revealed that hypertension was diagnosed earlier in subjects with mutation than in subjects without. In several studies, the role of genetic factors in the early development of hypertension has been shown [30, 31]. Our findings are in agreement with a study suggesting a genetic background in the pathogenesis of hypertension. However, it is difficult to exclude the role of environmental fator/s which may be more relevant than the *NOD2* mutation. Hence question arises whether mutation 3020insC predisposes to the development of hypertension or not. Taking into account the results of the previous study on preeclampsia and the role of innate immunity in vascular damage, it appears very likely [3–6, 9, 23]. Unfortunately, our study has not solved this problem. Moreover, our study has some limitations. Firstly, the sample of subjects with *NOD2* mutation was too small to drawn firm conclusions. Secondly, we do not know the frequency of the 3020insC mutation in the population without hypertension, and in the face of this fact, it is difficult to prove whether this mutation plays a role in the pathogenesis of hypertension.

Conclusions

The 3020insC mutation in the *NOD2* gene occurs among hypertensives. It suggests also that the 3020insC mutation may play a role in arterial remodelling in hypertensive patients.

References

1. Barenbrock M., Hausberg M., Kosch M. *et al.* Flow-mediated vasodilation and distensibility in relation to intima-media thickness of large arteries in mild essential hypertension. Am. J. Hypertens. 1999; 12: 973–979.
2. Lee K.W., Blann A.D., Lip G.Y. High pulse pressure and nondipping circadian blood pressure in patients with coronary artery disease: relationship to thrombogenesis and endothelial damage/dysfunction. Am. J. Hypertens. 2005; 18: 104–115.
3. McDermott M.F., Tschopp J. From inflamasomes to fevers, crystals and hypertension: how basic research explains inflammatory disease. Trends. Mol. Med. 2007; 13: 381–388.
4. Pickup J.C., Mattock M.B. Activation of the innate immune system as a predictor of cardiovascular mortality in type-2 diabetes mellitus. Diabet. Med. 2003; 20: 723–726.
5. Mazzolai L., Duchosal M., Korber M. *et al.* Endogenous angiotensin II induces atherosclerotic plaque vulnerability and eliecits a Th1 response in apoE-/- mice. Hypertension 2004; 44: 277–282.
6. Hansson G.K., Libby P., Schonbeck U. Innate and adaptive immunity in the pathogenesis of atherosclerosis. Circ. Res. 2002; 91: 281–291.
7. Medzhitov R., Janeway C. Innate immunity. N. Engl. J. Med. 2000; 343: 338–344.
8. Libby P., Ridker P.M., Maseri A. Inflammation and atherosclerosis. Circulation 2002; 105: 1135–1134.
8. Schnare M., Rollinghoff M., Qureshi S. Toll-like receptors:sentinels of host defence against bacterial infection. Int. Arch. Allergy Immunol. 2006; 139: 75–85.
9. Guha M., Mackman N. LPS induction of gene expression in human monocytes. Cell. Signal. 2001; 13: 85–94.
10. Hugot J-P., Chamaillard M., Zouall H. *et al.* Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 2001; 411: 599–603.
11.Ogura Y., Bonen D.K., Inohara M. *et al.* A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 2001; 411: 603–606.
12. Bertin J., Nir W-J., Fischer C.M. *et al.* Human CARD4 protein is a novel CED-4/Apaf-1 cell death family member that activates NF-kappaB. J. Biol. Chem. 1999; 274: 12955–12958.
13. Chen F., Castranova V., Shi X., Demers L.M. New insights into the role of nuclear factor-kB, a ubiquitous transcription factor in the initiation of disease. Clin. Chem. 1999; 45: 7–17.
14. Kobayashi K., Inohara N., Hernandez L.D. *et al.* RICK/ /Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune system. Nature 2002; 416: 194–199.
15. Karin M. How NFkappaB is activated: The role of the IKK complex. Oncogene 1999; 18: 6867–6874.
16. Sondka Z., Tretyn A., Szeliga J., Jackowski M. Involvement of leucine rich repeats (LRR) domain containing proteins in molecular mechanisms of innate immunity of plants and animals. Post. Biol. Komorki 2006; 33: 635–655.
17. Intengan H.D., Schiffrin E.L. Vascular remodeling in hypertension. Role of apoptosis, inflammation, and fibrosis. Hypertension 2001; 38 (part 2): 581–587.
18. Holler E., Rogler G., Herfarth H. *et al.* Both donor and recipient NOD2/CARD15 mutations associate with transplant-related mortality and GvHD following allogenic stem cell transplantation. Blood 2004; 104: 889–894.
19. Miceli-Richard C., Lesage S., Rybojad M. *et al.* CARD15 mutations in Blau syndrome. Nature. Genet. 2001; 29: 19–20.
20. Szeliga J., Sondka Z., Jackowski M. *et al.* The outline of immunopathogenesis of Crohn's disease with special consideration of NOD2/CARD15 gene polymorphism. Gastroenterol. Polska 2007; 14: 129–133.

21. Davey M.P., Martin T.M., Planck S.R. *et al.* Human endothelial cells express NOD2/Card15 and increase IL-6 secretion in response to muramyl dipeptide. Microvasc. Res. 2006; 71: 103–107.
22. Oh H-M., Lee H-J., Seo G-S. *et al.* Induction and localization of NOD2 protein in human endothelial cells. Cell. Immunol. 2005; 237: 37–44.
23. Van Rijn B.B., Franx A., Steegers E.A.P. *et al.* Maternal TLR4 and NOD2 variants, proinflamatory phenotype and susceptibility to early-onset preeclampsia and HELLP syndrome. PLoS ONE 2008; 3: e1865.
24. Kurzawski G., Suchy J., Kładny J. *et al.* The NOD2 3020insC mutation and risk of colorectal cancer. Cancer Res. 2004; 64: 1604–1606.
25. Lubiński J., Huzarski T., Kurzawski G. *et al.* The 3020insC Allele of NOD2 predisposes to cancer of multiple organs. Heredit. Cancer Clin. Pract. 2005; 3: 59––63.
26. Szeliga J., Sońdka Z., Jackowski M. NOD2/CARD15 polymorphism in patients with recital cancer. Med. Sci. Monit. 2008; 14: CR480–484.
27. Kazanawa N., Okafuji I., Kambe N. *et al.* Early-onset sarcoidosis and CARD15 mutations with constitutive nuclear factor kappa-B activation: common genetic etiology with Blau syndrome. Blood 2005; 105: 1195–1197.
28. Weber K.T. From inflammation to fibrosis: a stiff stretch of highway. Hypertension 2004; 43: 716–719.
29. Wernig F., Mayr M., Xu Q. Mechanical stretch-induced apoptosis in smooth muscle cells is mediated by integrin signaling pathways. Hypertension 2003; 41: 903–911.
30. Hunt S.C., Williams R.R., Barlow G.K. A comparison of positive family history definitions for defining risk of future disease. J. Chron. Dis. 1986; 39: 809–821.
31. Hunt S.C., Ellison R.C., Atwood L.D. *et al.* Genome scans for blood pressure and hypertension: the National Heart, Lung, and Blood Institute Family Heart Study. Hypertension 2002; 40; 1–6.