Abstract

**Background:** Epidemiological observations suggest that cancer arises from chronically inflamed tissues. Inflammatory bowel disease (IBD) is a typical example as patients with longstanding IBD are at an increased risk for developing colorectal cancer (CRC) and mutations of the **NOD2/CARD15** gene increase the risk for Crohn’s disease (CD). Recently, **NOD2/CARD15** has been associated with a risk for CRC in some studies, which stemmed from ethnically diverse populations. Our aim was to identify common **NOD2/CARD15** mutations in Hungarian patients with sporadic CRC.

**Methods:** A total of 194 sporadic CRC patients (m/f: 108/86, age at diagnosis of CRC: 63.2 ± 9.1 years old) and 200 healthy subjects were included. DNA was screened for SNP8, SNP12 and SNP13 **NOD2/CARD15** mutations by denaturing-HPLC and confirmed by direct sequencing.

**Results:** **NOD2/CARD15** mutations were found in 28 patients (14.4%) and in 23 controls (11.5%, p = NS). Allele frequencies for SNP8/R702W (1.8% vs. 1.5%) SNP12/G908R (1.8% vs. 1.8%) and SNP13/3020insC (3.6% vs. 2.5%) were also not statistically different between patients and controls. The clinicopathologic characteristics of CRC patients with or without **NOD2/CARD15** mutations were not significantly different.

**Conclusion:** Our results suggest that common **NOD2/CARD15** mutations alone do not contribute to CRC risk in the Hungarian population.
lion patients die of the disease each year [1]. In Hungary, CRC mortality has almost tripled in the past four decades, with a great proportion of the patients being diagnosed only in advanced stages [2]. The pathogenesis of sporadic CRCs is thought to be multifactorial, with multiple genetic and various environmental factors involved [3-5].

Epidemiological studies have suggested that chronic continuous inflammation predisposes to cancer [6]. A typical example is the association between inflammatory bowel diseases (IBD) and colorectal cancer (CRC) [7]. Although CRC, complicating ulcerative colitis and Crohn's disease, accounts for only 1–2% of all cases of CRC in the general population, it is considered a serious complication of the disease accounting for 1 in 6 of all deaths in IBD patients [8,9]. Precursor lesions of CRC may often have inflammatory histological features [10]. Inflammation may favour tumorigenesis by inducing DNA damage [11] stimulating continuous cell proliferation or apoptosis [12] and stimulating angiogenesis.

Assuming that the underlying chronic inflammation in IBD may be implicated in the progression of CRC, genetic factors, known to be involved in the chronic inflammatory process in ulcerative colitis and Crohn’s disease, may simultaneously hasten the development of CRC in IBD patients. Several studies have shown that NOD2/CARD15, a gene that overlaps with the IBD1 locus on chromosome 16q12, is significantly associated with susceptibility to IBD [13]. The physiological role of the CARD15/NOD2 protein remains under detailed examination. It is a cytoplasmic protein expressed in peripheral blood monocytes, Paneth cells and intestinal epithelial cells, and is structurally related to the well-described R proteins in plants, which mediate host resistance to microbial pathogens [14]. Variant alleles result in altered NF-κB activity [15,16]. Variant NOD2/CARD15 alleles are also associated with reduced α-defensin secretion from Paneth cells in response to bacteria [17]. Finally, NOD2/CARD15 was found to be involved in the regulation of TLR2 [18] and carriers of the variant alleles exhibited increased intestinal permeability [19].

Two single-nucleotide polymorphisms of NOD2/CARD15 (SNP8: R702W and SNP12: G908R) and a frameshift mutation (SNP13: 3020insC) were shown by independent groups to be associated with susceptibility to CD [20,21]. The presence of one variant allele increases the risk of developing CD 1.5–4.3-fold, and of two copies by up to 20–40-fold [22,23], yet rates are lower in Northern Europe [24].

Recently, NOD2/CARD15 was found to increase the risk for colorectal cancer (CRC). Kurzawski et al. [25] found that the presence of 3020insC mutation increased the risk of developing CRC by 2.23-fold in Polish patients with an older average age at diagnosis. This was however not confirmed in a Finnish study by Alhopuro et al. [26]. Noteworthy, in the most recent Greek study, all three common variants were found to be associated with an increased risk for CRC (OR: 2.4–5.2) [27].

In light of these findings, and given that the frequency of the mutations varies in different populations, our aim was to investigate the presence of the three common NOD2/ CARD15 variants in a large cohort of patients with sporadic CRC in Hungary, a country with a high CRC incidence rate, comparable to that observed in Poland.

**Methods**

One hundred and ninety-four consecutive Caucasian patients with sporadic CRC were investigated (male/ female: 108/86, age at diagnosis of CRC: 63.2 ± 9.1 years old). All patients with known hereditary cancer syndromes and previous diagnosis of IBD or a positive family history of CRC were excluded. The clinical data, symptoms (hematochezia, weight loss, anemia, changes in bowel movement habits) and clinicopathologic characteristics of the patients are shown in Table 2.

The control group for mutation analysis consisted of 200 gender-matched healthy Caucasian subjects (male/ female: 102/98), without any known gastrointestinal disease. Also colorectal cancer was absent in the family history of controls [23]. The study was approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics. Each patient was informed on the nature of the study and signed the informed consent form.

**Detection of NOD2/CARD15 SNP8, SNPl2 and 13 mutations**

Genomic DNA was isolated from whole blood according to the QIAmp DNA blood mini kit (QIAGEN GmbH, Germany). Each exon was amplified by PCR using previously published primer sequences [23]. The initial denaturation step (at 94°C for 7 min) was necessary in order to activate the AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), followed by 33 cycles (at 94°C for 20 s, at 61°C for 30 s, at 72°C for 25 s) with a final extension step at 72°C for 7 min. Then, denaturing high-performance liquid chromatography (dHPLC, wave DNA fragment analysis system, Transgenicom Limited, UK) was performed to analyze the exons. In the final step, PCR products were denatured at 94°C for 5 min to induce heteroduplex formation during the subsequent step of slowly cooling down to room temperature, over thirty minutes. Five microliters of these PCR products was then automatically loaded onto the DNASep cartridge (Transgenicom Limited, UK) in the wave system. The specific acetonitrile
gradient to elute each exon was established by using the WaveMaker 3.4.4 software. The particular run temperature for the detection of each SNP was determined using positive controls, which were kindly provided by Dirk Seegert from Kiel, Germany.

Finally, when a sequence variation was observed in the dHPLC profile, the relevant PCR product was sequenced on both strands to confirm the alteration. Sequencing reactions were performed with the ABI BigDye terminator cycle sequencing kit v1.1 (Applied Biosystems, Foster City, CA, USA) and samples were sequenced on an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

**Statistical analysis**

Variables were tested for normality by Shapiro Wilk’s W test. T-test with separate variance estimates, χ²-test and χ²-test with Yates correction were used to test differences between patients with CRC and controls, as well as within subgroups of CRC patients. Odds ratios (OR) and logistic regression were calculated to compare genetic and clinical data. A p value < 0.05 was considered statistically significant. For the statistical analysis, SPSS 12.0 (SPSS Inc, Chicago, IL, USA) was used with the help of a statistician (Dr. Peter Vargha).

**Results**

A large number of CRC patients were diagnosed at an advanced stage (T stage III-IV: 76.2%) and with clinical symptoms (57.7%) as shown in Table 2. NOD2/CARD15 mutations were found in 28 patients (14.4%) and in 23 controls (11.5%), p = 0.45, OR: 1.29 (95%CI: 0.72–2.34). Allele frequencies for SNP8/R702W (1.8% vs 1.5%), SNP12/G908R (1.8% vs. 1.8%) and SNP13/3020insC (3.6% vs. 2.5%) were also not statistically different between CRC patients and controls (see Table 1). All patients and controls were heterozygous for a particular mutation, no homozygous or compound heterozygous carriers were identified.

There was no difference between the age at diagnosis in the group of patients harboring at least one mutation (mean age 64.5 ± 7.9 years old) compared to the patients without the mutations (mean age 63.0 ± 9.3 years old). Furthermore, the CARD15/NOD2 variants carrier status was not associated with any of the examined clinicopathologic variables (Table 2).

**Discussion**

Contrary to previous reports, in the present study we were unable to detect an association between the prevalence of common NOD2/CARD15 mutations and the risk for sporadic CRC in a Hungarian population. Comparable variant allele frequencies were observed in both, the patient and control groups.

A possible explanation for the differences between the previous studies and the present one may be the differences in carrier frequencies among the controls. An association between NOD2/CARD15 was initially reported by a Polish study in 2004 [25]. The incidence of CRC in Poland is high, similar to Hungary. An association between the 3020insC mutation and the risk for CRC (OR: 2.23) was reported in 250 Polish CRC patients older than 50 years, at the time of time diagnosis. The carrier frequency was 14.4% in this subgroup of patients, however, it was only 7% in controls. This was not confirmed in patients who were younger than 50 years, at the time of diagnosis or in patients with HNPCC or a family history of CRC. In addition, no other common NOD2/CARD15 mutations were examined in the study.

In contrast, no association was found between the above mutation and the risk for CRC cases with or without family history in 926 CRC cases analyzed in a Finnish study. We have to note though, that the carrier (3.7%) as well as allele frequencies (1.9%) for both CRC patients and controls was lower in the Finnish study compared to the previous study as well as the present one. Variant allele frequencies in Finland (which by language is related to
Hungary) were comparable to those previously reported in IBD patients [24]. The prevalence of R702W, G908R and 3020insC was 3.3%, 0.6% and 4.8%, respectively, in the Finnish IBD study (in controls it was 1.8%, 0 and 1.7%) with only 3020insC being more common in CD compared to controls. Noteworthy, unlike in the Polish study in the study by Alhopuro et al. [26] patients with an age older than 50 years at diagnosis did not have an increased frequency of 3020insC mutation (4.3%) compared to patients with an age ≤50 years at the time of CRC diagnosis. In addition, no differences in the variant NOD2/CARD15 alleles were found between with any clinicopathological characteristics.

The more recent study, from Greece, reported an association between all three common NOD2/CARD15 variants and the risk for sporadic CRC in 104 consecutive patients and 100 controls. Carrier frequencies in CRC patients were surprisingly high; 23% for 3020insC, 9.6% for R702W and 13% for G908R compared to much lower rates in the controls (12%, 2% and 7%, respectively). In addition, carrier rates in CRC patients were comparable to the rates previously reported in Greek IBD patients [28]. Nonetheless, variant allele frequencies were much higher compared to the present study. In the most recent study by Roberts et al [29], not only SNP8 (OR: 2.3) were associated with the risk of sporadic CRC but also the presence of any common variant alleles (OR:2.8, 95%CI: 1.5–5.4). In addition, two homozygous SNP8 carriers were detected in the CRC patient group. Male gender was associated to the carriage of variant allele.

In concordance with a previous report from Finland [26], we did not find any association between clinicopathological characteristics or the location of the CRC and the presence of NOD2/CARD15 variants. In contrast, in the Greek study [27], an association was found between the TNM stage at diagnosis and the presence of the variant NOD2/CARD15 allele. NOD2/CARD15 variant alleles were associated with a more advanced TNM stage; however, partly due to the small number of patients, results were not corrected for other possible confounding factors (e.g. age at diagnosis or tumor differentiation). Finally, we have to note that according to previously published data (Polish-Greek study) a patient number of between 175–280 would be necessary to detect the reported association with a type I error of 0.05 and a type II error of 0.10, thus the present study had enough statistical power to detect the above differences in NOD2/CARD15 polymorphisms if present.

**Conclusion**

In summary, common NOD2/CARD15 mutations were not associated with disease susceptibility for sporadic CRC in a Hungarian population. This, in concordance with previous reports, suggests that it is unlikely that NOD2/CARD15 mutations alone are responsible for the development of sporadic CRC.

---

**Table 2: Clinicopathologic characteristics of colorectal cancer patients (CRC) with respect to the presence or absence of NOD2/CARD15 mutations**

| Characteristic                  | Total (n = 194) | Carrier (n = 28) | Non-carrier (n = 166) |
|--------------------------------|-----------------|-----------------|-----------------------|
| Male/female                     | 108/86          | 17/11           | 91/75                 |
| Age at diagnosis (years)        | 63.2 ± 9.1      | 64.5 ± 7.9      | 63.0 ± 9.3            |
| Age ≤60 years                   | 63              | 9               | 54                    |
| Age >60 years                   | 131             | 19              | 112                   |
| Symptoms at diagnosis*          |                 |                 |                       |
| Yes                            | 112             | 20              | 92                    |
| No                             | 82              | 8               | 74                    |
| Tumor location                  |                 |                 |                       |
| Rectum                         | 83              | 6               | 77                    |
| Left-colon                     | 73              | 15              | 58                    |
| Right colon                    | 38              | 7               | 31                    |
| T stage                         |                 |                 |                       |
| 1                              | 6               | 2               | 4                     |
| 2                              | 40              | 6               | 34                    |
| 3                              | 113             | 15              | 98                    |
| 4                              | 35              | 5               | 30                    |
| N stage#                       |                 |                 |                       |
| 0                              | 63              | 8               | 55                    |
| 1 or 2                         | 86              | 10              | 76                    |

*symptoms: hematochezia, weight loss, anemia, changes in bowel movement habits
# data available in 149 patients
p = not significant, by χ² or T-test with separate variance estimates as appropriate
Competing interests
The author(s) declare that they have no competing interests.

Authors’ contributions
PLL: design of the study, collected patients, statistical analysis and drafted the manuscript; EH, FSZ, PF, LL, SF, JO, OG and JP: collected patients, reviewed the manuscript; GV: statistical analysis and drafted the manuscript; KZ: performed the DNA analysis, reviewed the manuscript; PF: design of the study, performed the DNA analysis, helped in drafting the manuscript. All authors read and approved the final manuscript.

References
1. Parkin DM: Global cancer statistics in the year 2000. Lancet Oncol 2001, 2:533-543.
2. Fuszek P, Horvath HC, Speer G, Papp A, Haller P, Fischer S, Halasz J, Jaray B, Szekely E, Schaff Z, Papp A, Bursics A, Harasanyi L, Lukovich P, Kupcsik P, Hitre E, Lakatos PL: Location and Age at Onset of Colorectal Cancer in Hungarian Patients between 1993–2004. The High Number of Advanced Cases Supports the Need for a Colorectal Cancer Screening Programme in Hungary. Anticancer Res 2006, 26:527-32.
3. Lakatos PL, Lakatos L: Current concepts on the genetics of hereditary and sporadic colorectal cancer and the role of genetics in the patient management. Orv Hetil 2006, 147:449-56.
4. Popat S, Wort R, Houlston RS: Interrelationship between microsatellite instability, thymidine synthase expression, and p53 status in colorectal cancer: implications for chemoresistance. BMC Cancer 2006, 6:150.
5. Fuszek P, Lakatos P, Tabak A, Papp J, Nagy Z, Takacs I, Horvath HC, Lakatos PL, Speer G: Relationship between serum calcium and Ca19-9 levels in colorectal cancer. World J Gastroenterol 2004, 10:1850-52.
6. Balkwill F, Mantovani A: Inflammation and cancer: back to Virchow? Lancet 2001, 357:539-45.
7. Rhodes JM, Campbell BJ: Inflammation and colorectal cancer: IBD associated and sporadic cancer compared. Trends Mol Med 2002, 8:10-6.
8. Eaden JA, Abrams KR, Mayberry JF: The risk of colorectal cancer in ulcerative colitis: a meta-analysis. Gut 2001, 48:526-35.
9. Lakatos L, Mester G, Erdelyi Z, David G, Pandur T, Balogh M, Fischer S, Varga P, Lakatos PL: Risk factors for ulcerative colitis-associated colorectal cancer in a Hungarian cohort of ulcerative colitis patients. Results of a population-based study. Inflamm Bowel Dis 2006, 12:205-11.
10. Higaki S, Akazawa A, Nakamura H, Yama H, Yoshida T, Okita K: Metaplastic polyp of the colon develops in response to inflammation. J Gastroenterol Hepatol 1999, 17:709-14.
11. Jaiswal M, LaRusso NF, Burgart LJ, Gores GJ: Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependent mechanism. Cancer Res 2000, 60:1844-90.
12. Sipos F, Molnar B, Zagony T, Berzsi L, Tulassay T: Growth in Epithelial Cell Proliferation and Apoptosis Correlates Specifically to the Inflammation Activity of Inflammatory Bowel Diseases: Ulcerative Colitis Shows Specific p53- and EGFR Expression Alterations. Dis Colon Rectum 2005, 48:775-86.
13. Lakatos PL, Fischer S, Lakatos L, Gal I, Papp J: Current concept on the pathogenesis of IBD: crosttalk between genetic and microbial factors. Pathogenic bacteria, altered bacterial sensing or changes in mucosal integrity take "toll"? World J Gastroenterol 2006, 12:1829-40.
14. Berrebi D, Maudinas R, Hugot JP, Camaillard M, Charreyre F, De Lagusie P, Yang C, Desreumaux P, Giovannini M, Cezard JP, Zouali H, Emilie D, Pechmair M: CARD15 gene overexpression in mononuclear and epithelial cells of the inflamed Crohn’s disease colon. Gut 2003, 52:840-864.
15. Bonen DK, Ogura Y, Nicolaes DL, Inohara N, Saab L, Tanabe T, Chen FF, Foster SJ, Duerr RH, Brant SR, Cho JH, Nunez G: Crohn’s disease-associated NOD2 variants share a signalling defect in response to lipopolysaccharide and peptidoglycan. Gastroenterology 2003, 124:140-146.
16. Maeda S, Hsu LC, Liu H, Bankston LA, Imura M, Kagnoff MF, Eckmann L, Karin M: NOD2 mutation in Crohn’s disease potentiates NF-kB activity and IL-1b processing. Science 2005, 307:734-738.
17. Wehkamp J, Harder J, Weichenthal M, Schwab M, Schaffeler E, Schlee M, Herrlinger KR, Stallmach A, Noack F, Fritz P, Schroder JM, Bevin CL, Fellermann K, Stange EF: NOD2 (CARD15) mutations in Crohn’s disease are associated with diminished mucosal adhesion expression. Gut 2004, 53:1658-1664.
18. Netea MG, Kullberg BJ, de Jong DJ, Franke B, Spreng T, Naber TH, Drent JP, Van der Meer JW: NOD2 mediates anti-inflammatory signals induced by TLR2 ligands: implications for Crohn’s disease. Eur J Immunol 2004, 34:2052-9.
19. Lopes S, Buning C, Grootscholten D, Dignass A, Kuechler I, Krueger S, Schmidt HH, Locs H: Genetic basis for increased intestinal permeability in families with Crohn’s disease: role of CARD15/NOD2 2001insC mutation? Gut 2006, 55:342-7.
20. Hugot JP, Camaillard M, Zouali H, Lesage S, Cezard JP, Belaiche J, Almer S, Tycko B, O’Morain CA, Gasior M, Bind F, Vinel JF, Corrot A, Modigliani R, Laurent-Puig P, Gower-Rousseau C, Macry J, Colombel JF, Sahibotou M, Thomas G: Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn’s disease. Nature 2001, 411:599-603.
21. Ogura Y, Bonen DK, Inohara N, Nicolaes DL, Chen FF, Ramos R, Britton H, Moran T, Kariluskaus R, Duerr RH, Achkar JP, Brand SR, Bayless TM, Kirschner BS, Hanauer SB, Nunez G, Cho JH: A frameshift mutation in NOD2 associated with susceptibility to Crohn’s disease. Nature 2001, 411:603-607.
22. Ahmad T, Armuzzi A, Bunce M, Mulcahy-Hawes K, Marshall SE, Orchard TR, Crawshaw J, Large O, de Silva A, Cook JT, Barnardo M, Cullen S, Welsh KI, Jewell DP: The molecular classification of the clinical manifestations of Crohn’s disease. Gastroenterology 2002, 122:854-866.
23. Lakatos PL, Lakatos L, Szalay F, Willheim-Polli C, Osterreicher C, Tulassay Z, Molnar T, Reinisch W, Papp J, Mozsk K, Ferenci P: Toll-like receptor 4 and NOD2/Card15 mutations in Hungarian patients with Crohn’s disease: phenotype-genotype correlations. World J Gastroenterol 2005, 11:4333-35.
24. Helio T, Halme L, Lappalainen M, Fogstad H, Paavola-Sakki P, Turnunen U, Farkkila M, Kruisie T, Kontula K: CARD15/NOD2 gene variants are associated with familial occurring and complicated forms of Crohn’s disease. Gut 2003, 52:558-562.
25. Kurzawinski G, Suchy J, Kladny J, Grabowska E, Mierzejewski M, Jakubowska A, Debiakni T, Cybulski C, Kowalska E, Szych Z, Domagala W, Scott RJ, Lubinski J: The NOD2 2010insC mutation and the risk of colorectal cancer. Cancer Res 2004, 64:1604-6.
26. Alhopurto P, Alvenainen T, Mecklin JP, Juhola M, Jarvinen HJ, Karhu A, Aaltoinen L: NOD2 2010insC is not sufficient for colorectal cancer predisposition. Cancer Res 2004, 64:7425-7.
27. Papanastasinou I, Theodoropoulos G, Gazouli M, Panoussopoulos D, Mantzaris GJ, Feketsouras E, Bramis J: Association between mutations in the CARD15/NOD2 gene and colorectal cancer in a Greek population. Int J Cancer 2005, 111:149-155.
28. Gazouli M, Mantzaris G, Kotsinas A, Zacharatos P, Papalambros E, Archimandritis A, Ikonomopoulos J, Gorgoulis VG: Association between polymorphisms in the toll-like receptor4, CD14 and CARD15/NOD2 gene and the risk of colorectal cancer. World J Gastroenterol 2005, 11:7245-7.
29. Roberts RL, Geary RB, Allington MDE, Morrin HR, Robinson BA, Frizzle FA: Caspase Recruitment Domain-Containing Protein 15 Mutations in Patients with Colorectal Cancer. Cancer Res 2006, 66:2532-2535.

Pre-publication history
The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-2407/7/54/prepub