

**NOD2 3020insC Alone Is Not Sufficient for Colorectal Cancer Predisposition**

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**Abstract**

Mutations in **NOD2** have been shown to associate with increased susceptibility to Crohn’s disease. A recent Polish study linked the truncating **NOD2 3020insC** variant with an increased risk of colorectal cancer (CRC) at an older age (>50 years) of disease onset, with an odds ratio of 2.23. We studied the possible contribution of the **3020insC** variant to CRC risk in a series of 1,042 Finnish population-based patients from which 926 samples were successfully analyzed and in 348 anonymous cancer-free controls. The frequency of the **3020insC** mutation was 3.7% in both CRC patients (34 of 926, including 1 homozygote) and cancer-free controls (13 of 348; odds ratio, 0.98; confidence interval, 0.51–1.88). Contrary to the Polish study, there was no significant difference in the mutation rates between CRC patients >50 years of age (25 of 576; 4.3%) and controls in the present series. We studied respective tumor tissue DNAs of all patients displaying heterozygous **3020insC** changes for loss of heterozygosity. Loss of heterozygosity at **NOD2** was observed in only 1 of the 33 CRC samples. Our results suggest that **NOD2 3020insC** alone does not contribute to CRC risk. If this variant predisposes to CRC, additional factors not present in the Finnish population need to be involved.

**Introduction**

Genetic variation of **NOD2** has been shown to associate with increased susceptibility to Crohn’s disease in several independent studies, although in some populations no association has been observed (1–9). Three common mutations, two missense changes (Arg702Trp and Gly908Arg) and one frameshift change (3020insC→1007fs), located in the leucine-rich repeat (LRR) domain of the **NOD2** protein have been reported to be linked with the disease (1, 4). Association of the **3020insC** mutation with Crohn’s disease has also been detected in the Finnish population (8). The **NOD2** protein shows structural homology with both the apoptosis regulators and plant disease resistance gene products (10). However, the exact molecular function of **NOD2** is not thoroughly established. The protein consists of 2 NH₂-terminal caspase recruitment domains, 1 nucleotide-binding domain, and 10 COOH-terminal LRRs; the caspase recruitment domains are involved in activating nuclear factor-κB pathways, and the LRRs are involved in the recognition of bacterial components (10–12). The **NOD2 3020insC** variant, resulting in a Leu1007→Pro substitution in the 10th LRR followed by a premature stop codon, has been shown to be less active in response to bacterial lipopolysaccharides, which might generate an increased inflammatory response by the adaptive immune system in patients with Crohn’s disease (1).

A recent study suggested an association of the **NOD2 3020insC** variant with colorectal cancer (CRC) predisposition in a consecutive series of Polish CRC patients (13). The mutation frequency was, however, significantly higher only in a group of 250 patients >50 years of age at diagnosis, whereas in CRC patients ≤50 years of age, no difference was discerned when compared with healthy controls. Here we studied the frequency of the **NOD2 3020insC** mutation in a population-based series of 1,042 Finnish CRC patients and in 348 anonymous cancer-free blood donors. We also investigated possible loss of the wild-type **NOD2** allele in the respective cancer samples of all heterozygous cases.

**Materials and Methods**

**Patients.** A population-based series of 1,042 Finnish CRC patients was analyzed for **NOD2 3020insC**. Both normal and tumor tissue samples were collected prospectively at nine Finnish central hospitals between 1994 and 1998. This extensively documented material is described in more detail in previous works (14, 15). DNAs from 348 anonymous cancer-free Finnish blood donors were obtained from the Finnish Red Cross Blood Transfusion Service. Complete clinical data, including patient’s age at disease onset, family history, pathology reports, grade, and molecular data, including microsatellite instability status, and **MLH1** and **MSH2** mutation screening results of all microsatellite instability cases were available from all CRC patients.

**Mutation and Loss of Heterozygosity Analysis.** Detection of the 3020insC mutation was performed by amplifying normal tissue DNA by allele-specific polymerase chain reaction (PCR) as described by Ogura et al. (1). A positive control was used in each PCR. The results were confirmed by amplifying the samples again with the primers framing a 533-bp region around the insertion spot (1) and by sequencing the product using Applied Biosystems (Foster City, CA) 3730 BD3.1 sequencing chemistry and 5.1 sequencing analysis software (Applied Biosystems). Respective histologically verified tumor tissue DNA of all 33 samples heterozygous for the 3020insC variant was sequenced using the aforementioned primers to assess possible loss of heterozygosity (LOH). **NOD2** mutation 3020insC was used as a polymorphism to determine possible LOH (Fig. 1).

**Statistical Methods.** χ² test with Yates correction was used in analyzing the results. P < 0.05 was considered significant.

**Results and Discussion**

A total of 1,042 CRC samples were analyzed for the **NOD2 3020insC** variant, and results were successfully derived from 926 samples. The mutation frequencies were identical in CRC patients (34 of 926; 3.7%) and population controls (13 of 348; 3.7%). One CRC patient was homozygous; the rest of the **NOD2** mutation-positive cases were heterozygotes for the 3020insC variant.

To assess the possible contribution of loss of the wild-type **NOD2** allele to 3020insC-related tumorigenesis, we sequenced the respective tumor tissue DNAs of all of the patient samples displaying a heterozygous **NOD2** mutation. LOH was detected in only 1 of the 33 samples; thus, evidence to support involvement of **NOD2** in the genesis of these cancers was not obtained through this effort (Fig. 1).

To analyze the impact of CRC family history on mutation frequency, the 926 CRC cases were divided into two groups: patients
with no first-degree relatives with CRC were classified as nonfamilial cases \((n = 798)\); whereas patients with one to five first-degree relatives with CRC were classified as familial cases \((n = 128)\). The occurrence of the mutation was also analyzed according to microsatellite instability status (Table 1). Finally, the CRC patients were divided into three subgroups similar to those described by Kurzawski et al. \((13)\). At this point, 25 mutation-positive hereditary nonpolyposis CRC patients were excluded from the analysis, resulting in a total of 901 patients. In group 1 \((n = 576)\), all patients were \(>50\) years of age at the time of diagnosis and had, at the most, one first-degree relative with cancer. Group 2 \((n = 68)\) was identical to group 1, except that all patients were \(\leq 50\) years old at diagnosis of the disease. Group 3 \((n = 256)\) represented CRC patients with familial cancer aggregation; i.e., patients from families in which at least two other family members were diagnosed with CRC or other malignancies \((\text{ref. 13}; \text{Table 2})\).

Table 1 Frequency of the NOD2 3020insC mutation in controls and in CRC patients

<table>
<thead>
<tr>
<th>Cohorts and Subgroups</th>
<th>No. of subjects</th>
<th>No. with mutation (%)</th>
<th>Allele frequency (%)</th>
<th>(P^*)</th>
<th>OR (CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>348</td>
<td>13 (3.7)</td>
<td>1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRC patients</td>
<td>926</td>
<td>34 (3.7)</td>
<td>1.9</td>
<td>0.91</td>
<td>0.98 (0.51–1.88)</td>
</tr>
<tr>
<td>Nonfamilial CRC</td>
<td>798</td>
<td>31 (3.9)</td>
<td>2.0</td>
<td>0.97</td>
<td>1.04 (0.53–2.01)</td>
</tr>
<tr>
<td>Familial CRC</td>
<td>128</td>
<td>3 (2.3)</td>
<td>1.2</td>
<td>0.64</td>
<td>0.62 (0.17–2.21)</td>
</tr>
<tr>
<td>MSI CRC</td>
<td>111</td>
<td>4 (3.6)</td>
<td>2.3</td>
<td>0.82</td>
<td>0.96 (0.31–3.02)</td>
</tr>
<tr>
<td>MSS CRC</td>
<td>815</td>
<td>30 (3.7)</td>
<td>1.8</td>
<td>0.90</td>
<td>0.98 (0.51–1.91)</td>
</tr>
</tbody>
</table>

Abbreviations: MSI, microsatellite instable; MSS, microsatellite stable.

* \(\chi^2\) with Yates correction factor; group versus controls.

Similar frequencies of the NOD2 3020insC variant in a population-based series of individuals with CRC and in healthy controls indicate that either the mutation does not predispose to CRC or the risk of CRC conferred by the mutation is, at the most, modest. The Polish study observed an OR of 2.23 in CRC patients older than 50 years \((13)\). Despite sufficient power, our study was also negative for this patient group. The frequency of the 3020insC mutation was reported to be somewhat higher in Polish CRC patients, who match the criteria of hereditary nonpolyposis CRC \((\text{the exact criteria were not specified})\) but display no mutations in the mismatch repair genes, although the difference did not reach statistical significance \((13)\). However, in our series, there was no aggregation of the 3020insC mutation to CRC families. Whereas our results speak strongly against NOD2 involvement in CRC predisposition in Finns, we cannot exclude a role for NOD2 as a low penetrance CRC susceptibility gene in other populations, such as the Polish population. There might also be modifying factors other than the NOD2 3020insC variant conferring an elevated NOD2-related risk of CRC in the Polish population, but not in the Finnish population. These might include, for instance, effects of other as yet unknown predisposition alleles or environmental factors.

In summary, we have analyzed the incidence of the NOD2 3020insC variant in 926 CRC patients and 348 healthy controls. Mutation frequencies were identical in CRC patients and control individuals. No evidence of silencing of the second allele by LOH was obtained. Our results suggest that NOD2 has no major contribution to CRC risk in the Finnish population. However, it has been proposed that there is regional heterogeneity within Europe in the contribution of NOD2 variants to Crohn’s disease susceptibility, reflecting effects of differing founder populations \((16, 17)\). The observed overall mutation frequency indeed appears to be higher in Poland than in many other Western populations \((2, 4, 8, 16)\). Also, environmental factors and additional genetic factors vary greatly between populations. Thus, additional data from different populations are needed to determine whether the NOD2 3020insC mutation predisposes to CRC and, if so, to identify the additional determinants necessary for the increased susceptibility.

Table 2 NOD2 3020insC mutation in subgroups according to Kurzawski et al. \((13)\)

<table>
<thead>
<tr>
<th>CRC subgroups</th>
<th>No. of subjects</th>
<th>No. with mutation (%)</th>
<th>Allele frequency (%)</th>
<th>(P^*)</th>
<th>OR (CI) by (\chi^2) test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>577</td>
<td>25 (4.3)</td>
<td>2.3</td>
<td>0.78</td>
<td>1.17 (0.59–2.32)</td>
</tr>
<tr>
<td>Group 2</td>
<td>68</td>
<td>2 (3.0)</td>
<td>2.2</td>
<td>0.78</td>
<td>0.78 (0.17–3.54)</td>
</tr>
<tr>
<td>Group 3</td>
<td>256</td>
<td>6 (2.3)</td>
<td>1.2</td>
<td>0.46</td>
<td>0.62 (0.23–1.65)</td>
</tr>
</tbody>
</table>

NOTE. Groups are defined as follows: Group 1, CRC patients \(>50\) years of age with one other malignancy, at most, in the family; Group 2, CRC patients \(\leq 50\) years of age with one other malignancy, at most, in the family; and Group 3, CRC patients of all ages with familial cancer aggregation \((\text{i.e., two or more other malignancies in the family})\).

* \(\chi^2\) with Yates correction factor; group versus controls.
Acknowledgments

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References

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