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Original Article

Variants in the neuropeptide gene *NUCB2* as a possible biomarker for colorectal cancer

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Abstract: Colorectal cancer (CRC) is one of the leading causes of cancer-related mortality worldwide. Recent evidence suggests a potential role for the neuropeptide nesfatin-1, encoded by the *NUCB2* gene, in cancer development. This study aimed to analyze the association between the rs1330 and rs757081 variants of *NUCB2* and CRC in a Mexican population. A total of 780 individuals were included in a cross-sectional study: 397 patients with CRC and 383 healthy controls. The CT and TT genotypes of rs1330 were significantly associated with CRC (odds ratio [OR] = 0.6127, P = 0.0029; OR = 2.6574, P = 0.000, respectively), as were the CG and GG genotypes of rs707581 (OR = 0.6825, P = 0.019; OR = 2.6975, P = 0.000, respectively). Hardy-Weinberg equilibrium was not met for either variant (P = 0.000). Haplotype analysis indicated that the T/G haplotype conferred increased risk, while C/C had a protective effect. These findings support a potential role for *NUCB2* variants as biomarkers for CRC susceptibility in the Mexican population.

Keywords: Nucleobindin-2, nesfatin-1, colorectal cancer, allelic variant, single nucleotide polymorphism

Introduction

Cancer is the most common noncommunicable disease with the most death in the world and represents a major public health problem requiring priority attention [1]. According to GLOBOCAN, cancer ranks as the second leading cause of death worldwide. Its etiology is complex due to the involvement of genetic and environmental factors [2]. It can be caused by somatic or germline mutations, which lead to intracellular alterations that disrupt normal growth and apoptosis regulation [3]. In 2019, cancer was the third leading cause of mortality in Mexico, with approximately 53% of cancer-related deaths attributed to six predominant types: lung, colorectal, gastric, prostate, breast and pancreatic cancer [4]. Recently, it has been reported that one of the risk factors for developing cancer is having type 2 diabetes (T2D). In this regard, Pearson-Stuttard et al. [5] estimated that 5.6% of cancers diagnosed in 2012 were due to the combined effects of diabetes and a high body mass index (BMI), suggesting a clear association between the presence of these two conditions and the development of cancer. Some reports have shown that hyperinsulinemia caused by T2D favors proliferation, reduces apoptosis, and contributes to carcinogenesis in the colon and rectum. Hence, a T2D diagnosis may increase the risk for the development of colorectal, pancreatic, and breast cancers [6].

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NUCB2, identified by Oh-I et al. [7], belongs to the family of nucleobindin proteins. These proteins are characterized by EF-hand Ca^{2+}-binding domains and the ability to bind to DNA, which gives them several physiological functions. The *NUCB2* locus is at 11p15.1 and encodes for an immature polypeptide that is cleaved by prohormone convertase (PC) 1/3 and PC2 to yield three fragments, nesfatin-1 (residues 1-82), nesfatin-2 (residues 85-163), and nesfatin-3 (residues 85-163). Nesfatin-1 is an 82 amino acid polypeptide and has three regions: N23 or the N-terminus, M30 or the active region, and C29 or the C-terminus [7, 8]. Nesfatin-1 is considered to be a neuropeptide; it is highly expressed in several regions of the central nervous system such as the cortex, limbic system, thalamus, hypothalamus, brainstem, cerebellum, and preganglionic sympathetic and parasympathetic nerves of the spinal cord [7, 8]. However, nesfatin-1 is also expressed in peripheral tissues such as adipose tissue, pancreas, duodenum, colon, stomach, and ovaries. Several functions have been associated with nesfatin-1 and it is recognized as an anorexigenic peptide with antioxidant, anti-inflammatory, antihyperglycemic, and anti-apoptotic functions. Moreover, it has been associated with several diseases, including obesity, T2D, and cancer, among others [9-11]. Recently, studies had reported high *NUCB2* expression in mammary, prostate, colon, endometrial, and papillary thyroid tumors as well as renal cell carcinoma compared with adjacent non-cancerous tissue [12]. These findings suggest a clear relationship between nesfatin-1 expression and poor cancer prognosis, with the promotion of metastasis and shorter disease-free survival. There are several variants of interest for *NUCB2*, however, few are known about the effect of the variants in cancer. One of the variants majorly analyzed is the intronic variant rs1330, previously studied and associated with T2D, obesity, and dietary behaviors, both of them risk factors to cancer development [13-15]. On the other hand, the rs757081 coding sequence variant, generate a missense substitution at position 338 of the protein (p. Gln338Glu) and had been included in the Catalogue of Somatic Mutations in Cancer (COSMIC) with the genomic mutation ID COSV60373905 [16]; studied previously in rhabdomyosarcoma [17] and has also been associated with polycystic ovaries and blood pressure [18, 19]. Based on recent observations that *NUCB2* expression significantly influences cancer progression, we analyzed the association of the *NUCB2* variants rs1330 and rs757081 as possible markers of colorectal cancer (CRC), as well as their possible association with this malignancy in patients with DM2.

Materials and methods

Patients and samples

A total of 780 individuals were included in this cross-sectional, non-probability study: 397 patients diagnosed with non-familial CRC and 383 healthy individuals, considered as controls (**Figure 1**). DNA was extracted from peripheral blood using the Miller method [20]. The NanoDrop One/One C Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific. Waltham, Massachusetts, USA) was used to determine the concentration and assess purity. Aliquots of DNA (15 ng) were stored at -20°C until variant genotyping. In addition, risk factors and clinical characteristics of CRC were analyzed, including the presence of T2D.

Genotyping

The rs1330 and rs757081 variants were identified by allelic discrimination with the QuantStudio 5 Real-Time PCR System for Human Identification (Applied Biosystems by Thermo Fisher Scientific. Waltham, Massachusetts, USA). Two TaqMan® SNP Genotyping Assays, specifically C ___945737_10 and C ___226-1417_10 (Thermo Fisher Scientific. Waltham, Massachusetts, USA), were used according to the manufacturer's instructions to identify the rs1330 and rs757081 variants, respectively.

Statistical analysis

Statistical analysis was performed using SPSS Statistics version 25.0 (IBM Corp. Armonk, NY, USA). Qualitative variables are expressed as frequencies and percentages. The Kolmogorov-Smirnov test was used to analyze the normality of the quantitative variables. The Hardy-Weinberg equilibrium of the rs1330 and rs757081 variants was determined by using the SHEsis online software (http://analysis.bio-x.cn/myAnalysis.php) [21]. A p value < 0.05 was considered to indicate a statistically significant difference.

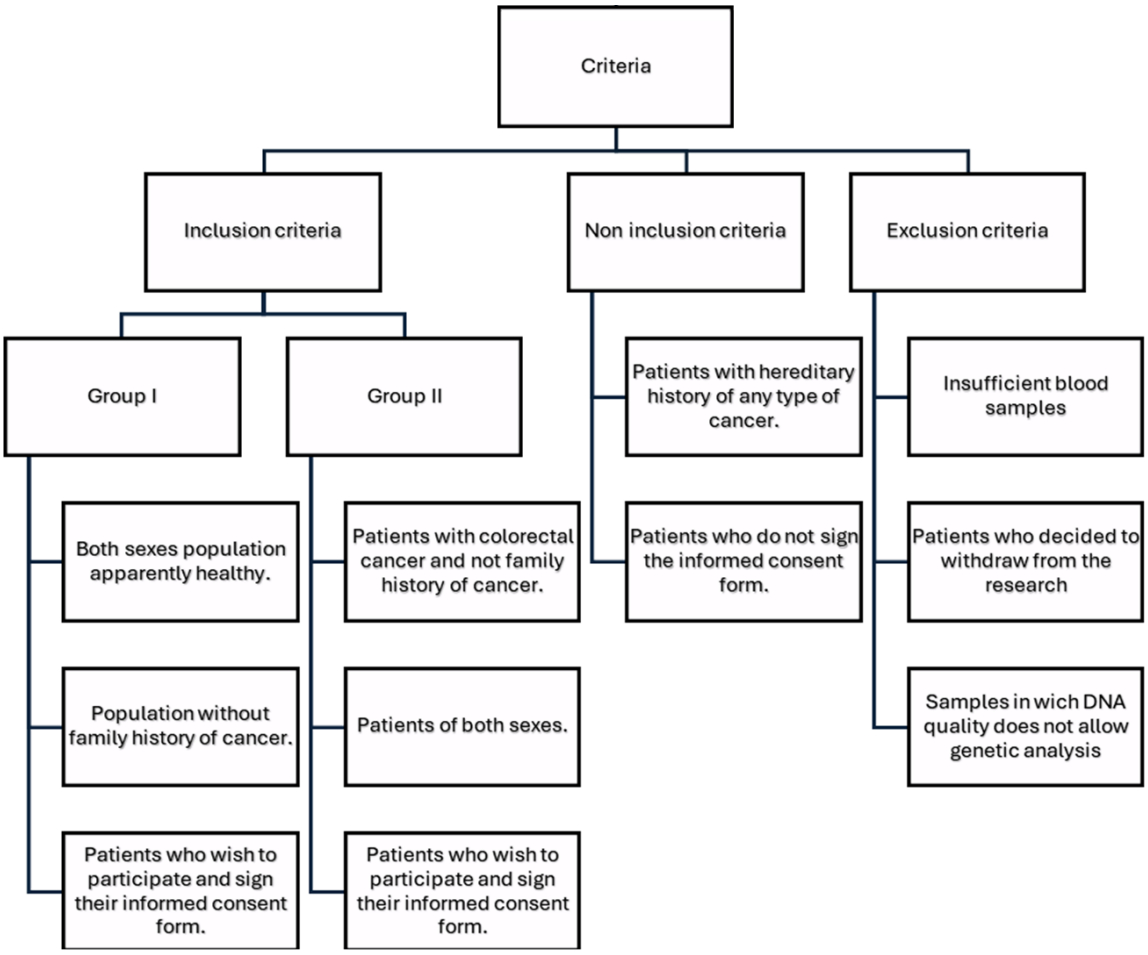

Figure 1. Inclusion and exclusion criteria for each group.

Ethical aspects

This project involved minimal risk to the participants, according to the General Health Law on Health Research in Mexico, and complies with the latest version (2016) of the World Medical Association's Declaration of Helsinki. The project was registered and approved by the Bioethics Committee of the Secretary of Health of the State of Jalisco (DGRPID/DI/CEI/04/23). All participants in the project signed an informed consent form.

Results

Table 1 shows the demographic variables and tumor characteristics of the patients. CRC was more frequent in men, and the average age at diagnosis of CRC was 61 years. The main tumor location was rectum-anus, followed by the left colon. Most of the tumors were stage III and poorly differentiated at the time of diagnosis. There were no significant differences in alcohol use, tobacco use, and BMI between the patients with CRC and the healthy controls.

Colon cancer and T2D

We found that 14.1% of the patients with CRC had also been diagnosed with T2D. In the patients with CRC and T2D, the average age of CRC diagnosis was 63 years, while in the patients without T2D, the average age of CRC diagnosis was 60 years. There were more men in both groups (55.4% and 54.5%, respectively). The most common site of CRC in patients with T2D was the rectum-anus, while in patients without T2D, it the descending colon. In patients with T2D, CRC was predominantly well-differentiated and there were no nodules at diagnosis (**Table 2**).

Table 1. Demographic characteristic of the patients with colorectal cancer (CRC) and healthy individuals (controls)

Variable	CRC group n = 397	Control group n = 383
Gender		
Female	180 (45)	174 (45)
Male	217 (55)	209 (55)
Age (years)	60.52 ± 10.48	59.55 ± 16.27
Body mass index		
Underweight	47 (12)	35 (9)
Normal weight	206 (52)	197 (51)
Overweight	114 (36)	141 (36)
Obesity	0	10 (2)
Tobacco use		
Yes	130 (32.7)	134 (35)
No	267 (67.3)	249 (65.0)
Alcohol use		
Yes	135 (34.0)	106 (27.7)
No	262 (66.0)	277 (72.3)
Type 2 diabetes		
Yes	56 (14.1)	
No	341 (85.9)	
Tumor localization		
Left colon	188 (47)	
Right colon	3 (1)	
Rectum-anus	206 (52)	
Stage		
I	10 (2)	
II	127 (32)	
III	169 (43)	
IV	91 (23)	
Differentiation		
Yes	67 (17)	
No	330 (83)	

Continuous variables are expressed as the mean ± standard deviation, while categorical variables are expressed as the frequency (percentage).

Genotyping in patients with CRC

The minor alleles of rs1330 and rs757081 of the *NUCB2* gene showed significant differences between the patients with CRC and the healthy controls (**Table 3**). The rs1330 and rs757081 allelic frequencies in the CRC group were not consistent with Hardy-Weinberg equilibrium (P = 0.000). The haplotype analysis for the rs1330 and rs757081 variants showed that the C/C haplotype is protective; in contrast, the T/G haplotype, which includes polymorphic alleles of both variants, has a risk effect (**Table 4**).

Discussion

There has been increased interest in the nesfatin-1 protein in cancer due to its involvement in the regulation of several pathways associated with cellular homeostasis in general. Although researchers have analyzed nesfatin-1 in different types of cancer, the available data are limited. In the present study, we analyzed the association between the rs1330 and rs757081 variants of *NUCB2* in patients with CRC. This disease is one of the most common malignancies worldwide, ranking third in both sexes, and was the second leading cause of death [22]. In our Mexican population, CRC had the same presentation as reported in other populations, with a higher incidence in men (55%) than women, consistent with GLOBOCAN [2]. Age also plays an important role in CRC: According to the National Cancer Institute (NCI) Surveillance, Epidemiology, and End Results (SEER) database, 67 years is the average age of diagnosis of CRC [23]. In our population, the average age of diagnosis was earlier at 60 years. This suggests the need for earlier screening policies in patients with risk factors for CRC. Risk factors such as tobacco use, alcohol consumption, and BMI did not differ between the patients with CRC and the healthy controls. One piece of relevant information in this study is the presence of T2D in 14.1% of patients with CRC, which is consistent with the estimated frequency in other populations [24]. The association between T2D and cancer has been reported previously; it is estimated that 30% of cancer cases occur in patients with T2D [25, 26]. It has been estimated that patients with T2D develop CRC almost 5 years earlier than the general population [27]. In addition, we found significant differences between patients with CRC and patients with CRC but not T2D: In the former group, most cases did not present nodules, and the average age of diagnosis was slightly later (63 years vs. 60 years for the group with CRC but not T2D).

Based on our *NUCB2* variant analysis, both rs1330 and rs757081 show strong associations with CRC, even as heterozygous and homozygous genotypes. These variants were

Table 2. Comparison of risk factors in patients with colorectal cancer (CRC) and with or without type 2 diabetes mellitus (T2DM)

Variable	CRC and T2DM n = 56 (%)	CRC without T2DM n = 341 (%)	*P* value
Gender			
Female	25 (44.6)	155 (45.5)	
Male	31 (55.4)	186 (54.5)	0.910
Alcohol use			
Yes	19 (33.9)	116 (34.0)	
No	37 (66.1)	225 (66.0)	0.990
Tobacco use			
Yes	14 (25.0)	116 (34.0)	
No	42 (75.0)	225 (66.0)	0.183
Tumor localization			
Left colon	16 (28.6)	172 (50.4)	
Rectum-anus	39 (69.6)	167 (49.0)	
Right colon	1 (1.8)	2 (0.6)	**0.008**
Stage			
I	2 (3.6)	8 (2.3)	
II	22 (39.3)	105 (30.8)	
III	23 (41.1)	146 (42.8)	
IV	9 (16.0)	82 (24.1)	0.433
Differentiation			
Yes	12 (21.4)	55 (16.1)	
No	44 (78.6)	286 (83.9)	0.326
Metastasis			
Yes	27 (48.2)	212 (62.2)	
No	29 (51.8)	129 (37.8)	**0.048**
BMI			
Underweight	4 (7.1)	43 (12.6)	
Normal weight	27 (48.2)	179 (52.5)	
Overweight	25 (44.6)	119 (34.9)	
Obesity	0 (0)	0 (0)	0.267

Continuous variables are expressed as the mean ± standard deviation, while categorical variables are expressed as the frequency (percentage). Categorical variables were analyzed using a chi-square test, while continuous variables were analyzed using Student's t-test. Values in bold indicate a significant difference ($P < 0.05$).

not in Hardy-Weinberg equilibrium, suggesting that they act more like mutations rather than variants. In this sense, it is important to consider that the rs757081 variant has recently been included in the Catalogue of Somatic Mutations in Cancer (COSMIC, number COSV60373905) and has been associated with T2D, polycystic ovaries, childhood adiposity, male obesity, and hypertension [18, 19, 28-31]. Our haplotype analysis confirmed the pathogenic effect of the T/G haplotype of rs1330 and rs757081 in patients with CRC from Mexico, increasing the risk 2.163 times. Conversely, the C/C haplotype, consisting of the wild-type alleles, showed a protective effect against CRC. The *NUCB2* genotypes did not show significant differences when comparing the patients with CRC and T2D to the patients with only CRC. It is important to highlight that, to the best of our knowledge, this is the first study to associate these variants with patients with CRC. However, previous studies have demonstrated that high *NUCB2* expression in several types of cancer is associated with a poor prognosis, favoring metastasis and poor disease-free survival. The authors of a study based on the isobaric tags for relative and absolute quantitation (*iTRAQ*)-based mass spectrometry in nasopharyngeal cancer tumors identified a total of 208 proteins where the nesfatin-1/NUCB2 protein was upregulated, demonstrating its implication in the development of this disease beyond metastasis or invasion into other tissues [32]. In a Chinese cohort of patients with breast cancer, Xie et al. [33] observed a significant association between high *NUCB2* expression in patients with CRC with stage III-IV malignant tumors but not in patients with stage I-II tumors. This strongly suggests that *NUCB2* is a cancer-associated oncogene and is associated with aggressive CRC progression.

In a study of breast cancer cells, nesfatin-1 showed higher expression in the cytoplasm compared with mastopathy ($P < 0.0001$), a phenomenon that was also confirmed in tissue samples from patients with ductal breast cancer. The authors also observed an inverse relationship between *NUCB2* expression and increased tumor grade, that is, when *NUCB2* expression is lowest, the tumor cells are poorly

Table 3. Comparison of the allelic and genotypic frequencies of the variants rs1330 and rs757081 of *NUCB2* gene in patients with colorectal cancer (CRC) and the healthy individuals (control group)

Variant rs1330	CRC group n = 397	Control group n = 383	OR (95% CI)	*P*-value
CC	172 (43)	169 (44)	1 (Reference)	-
CT	106 (27)	170 (44)	0.6127 (0.4438-0.8457)	**0.003**
TT	119 (30)	44 (12)	2.6574 (1.7710-3.9873)	**0.000**
CT+TT	225 (56.7)	214 (55.9)	1.0331 (0.7784-1.3710)	0.822
C	450 (57)	508 (66)	1 (Reference)	-
T	344 (43)	258 (34)	1.5052 (1.2258-1.8482)	**0.000**
Variant rs757081	CRC group n = 397	Control group n = 383	OR (95% CI)	*P*-value
CC	173 (44)	175 (46)	1 (Reference)	-
CG	112 (28)	166 (43)	0.6825 (0.4962-0.9387)	**0.019**
GG	112 (28)	42 (11)	2.6975 (1.7862-4.0737)	**0.000**
CG+GG	224 (57)	208 (54.3)	1.0894 (0.8213-1.4449)	0.553
C	458 (58)	516 (67)	1 (Reference)	-
G	336 (42)	250 (33)	1.5142 (1.2317-1.8615)	**0.0001**

The data are presented as the frequency (percentage). The odds ratio (OR) is presented with its 95% confidence interval (CI). The chi-square test was used for analysis. The most frequent genotype and allele for each variant in the population were used as references. Values in bold indicate a significant difference (P < 0.05).

Table 4. Haplotype analysis of the rs1330 and rs757081 *NUCB2* variants in patients with colorectal cancer (CRC) and healthy individuals (control group)

Haplotype rs1330/rs757081	CRC (n = 397)	Control Group (n = 383)	OR (95% CI)	*p*-value
C/G	173 (0.218)	170 (0.223)	0.976 (0.768-1.240)	0.841
C/C	277 (0.348)	337 (0.441)	0.679 (0.553-0.832)	**0.000**
T/G	162 (0.205)	81 (0.106)	2.163 (1.623-2.883)	**0.000**
T/C	181 (0.228)	177 (0.230)	0.989 (0.781-1.252)	0.926

Haplotype frequency was estimated with the SHEsis software. The wild allele was used as the reference. The odds ratio (OR) is presented with its 95% confidence interval (CI). Values in bold indicate a significant difference (P < 0.05).

differentiated. Other important factors were the ER+ and PR+ status (P = 0.0001), but not the HER2+ status; older age (> 66 years, P = 0.0136); and 5-year survival (P = 0.0186). Moreover, nesfatin-1 expression was lower in triple-negative breast cancer than in other subtypes [10].

According to Ning et al. [34], there is a clear positive relationship between higher nesfatin-1 expression and breast cancer tumors (P < 0.001) as well as poor survival (P < 0.05). In addition, analysis of the NUCB2 protein using the CUCKOO database revealed eight lysine residues as potential acetylation sites. They noted that the acetyltransferase cAMP-response element-binding protein (CREB) and the binding protein (CREBBP) were involved in 75% of the cases, while the acetyltransferase 2B (KAT2B) was involved in 25% of the cases. Researchers have proposed that nesfatin-1 overexpression drives breast cancer metastasis by upregulating the SREBP2 (Sterol Regulatory Element-Binding Protein 2) and 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGCR) proteins through to the mammalian target of rapamycin complex 1 (mTORC1) pathway, thereby increasing cholesterol synthesis. In gastric cancer, Ren et al. [35] analyzed the serum concentration levels of nesfatin-1 and observed that it was significantly increased compared with the control group (P < 0.001). In the same study, the authors observed that NUCB2/nesfatin-1 knockdown suppressed proliferation, migration, and epithelial-mesenchymal transition (ETM) via N-cadherin. In osteosarcoma, *NUCB2* overexpression favors cell progression and inhibits metabolic stress-in-

duced cell death by regulating C-X-C Motif Chemokine Ligand 8 (CXCL8) expression. In turn, CXCL8 overexpresses Programmed Cell Death 1 Ligand 1 (PD-L1), which leads to inhibition of CD8+ T lymphocytes. NUCB2 overexpression has been observed in human osteosarcoma samples, accompanied by CXCL8 and PD-L1 overexpression. These changes were associated with more pronounced metastasis and worse survival. These results demonstrate that *NUCB2* plays a fundamental role in the oncogenesis of osteosarcoma by regulating the immune response through the CXCL8/PD-L1 axis. NUCB2 was also shown to interact with Nuclear Casein Kinase and Cyclin Dependent Kinase Substrate 1 (NUCKS1), a member of a family of proteins that have been shown to play an important role in tumor development. It is suggested that in osteosarcoma, NUCB2 regulates the overexpression of NUCKS1 through ubiquitination [36].

Our study has two main limitations. First, we did not have enough information on the progression of the tumors to assess survival at 5 years. Second, it could be interesting to relate the results observed in this work to the *NUCB2* expression protein to analyze the impact of the variants.

Conclusion

We showed that the presence of the polymorphic T and G alleles of the rs1330 and rs-757081 variants of *NUCB2*, respectively, are associated with CRC in heterozygous or homozygous states in the Mexican population. They may serve as a potential biomarker for CRC.

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Disclosure of conflict of interest

None.

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