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Influence of *NECTIN1* Gene Variants in Herpesvirus Infection in Patients with Thyroid Nodules

Teixeira ES[1], Nascimento M[1], Peres KC[1], Leão SLS[1], Dal' Bó IF[1], Rabi LT[1,2], Almeida JFM[1,2], Barreto IS[3,4], Assumpção LVM[5], Tincani AJ[1], Bufalo NE[1,4,6] and Ward LS[1*]

[1]*Laboratory of Cancer Molecular Genetics, University of Campinas, Brazil*

[2]*Health Science Institute, Paulista University, Brazil*

[3]*Department of Pathology, University of Campinas, Brazil*

[4]*Department of Pathology, São Leopoldo Mandic Institute and Research Center, Brazil*

[5]*Department of Medicine, University of Campinas, Brazil*

[6]*Department of Medicine, Max Planck University Center, Brazil*

Correspondence should be addressed to Laura Sterian Ward, Laboratory of Cancer Molecular Genetics, School of Medical Sciences, PO Box 6111, Campinas, SP-Brazil

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ABSTRACT

Herpes viruses (HSV) have been implicated in the pathophysiology of thyroid nodules, but how they may be associated with malignancy is still poorly understood. In addition to allowing entry and replication of HSV cells, *Nectin1* cell adhesion molecules play an important role in cell proliferation, differentiation, and migration. Aiming to investigate a possible role of Nectin1 gene variants on the susceptibility to HSV and the development of thyroid cancer, we employed computational analysis to select single nucleotide polymorphisms that could modify nectin1 morphology and function. We further used real-time PCR to genotype 440 control individuals (137 men and 303 women, 38.09 ± 12.10 years old) and 440 thyroid nodule patients (72 men and 368 women, 40.40±10.3 years old) for *NECTIN1* rs199962982, rs14125361, and rs7940667. In addition, we screened 300 out of the 810 participants of the investigation, including 150 thyroid nodule patients (65 benign and 85 malignant) and 150 controls, for HSV-2 presence. Polymorphisms rs199962982 and rs141253617 did not show variation in genotype. The rs7940667 genetic profile was similar in thyroid nodule patients and controls ($p = 0.6000$), in benign and malignant thyroid nodules ($p = 0.4319$). However, Nectin1 rs7940667 variants were less frequent in tumors with capsular invasion ($p = 0.0105$). Anti-HSV-2 seroprevalence was similar in thyroid nodule patients and controls ($p = 0.5122$), in benign and malignant thyroid nodules ($p = 0.4522$) and had no association with the genotypic profile of *Nectin1*. A larger series of cases and functional studies may confirm a protective role of the *NECTIN1* variants against HSV viral infection and their relationship with thyroid cancer features.

KEYWORDS

Thyroid cancer; Aggressiveness; Viral infection; Single nucleotide polymorphism

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INTRODUCTION

Thyroid cancer (TC) is the most common malignant endocrine pathology worldwide and represented about 5.4% of all cancers detected in Brazilian women in 2020 [1]. The rapid growth in the number of cases has placed a considerable burden on the Brazilian health system [2]. Easy access to sensitive diagnostic methods, such as the ultrasound, in a growing elder population is undoubtedly the major factor of this increase in the TC diagnosis rate. However, solid evidence points to the contribution of other factors [3-5]. Viral infections are major oncogenic agents and may contribute to at least 20% of all human cancers [6] with about 2.2 million cancer diagnoses worldwide estimated to be associated with infectious agents, corresponding to an incidence of 25 cases per 100,000 people/year [7]. Several well-established oncogenic viruses and numerous global studies exploring the potential of infections in human malignancies already demonstrated the important role of herpes viruses. For instance, Epstein-Barr virus (EBV) is strongly linked to the risk of developing nasopharyngeal cancer and certain types of rapidly growing lymphomas, such as Burkitt's lymphoma [8].

Herpes simplex virus type 2 (HSV-2/HHV-2) infection is the most common worldwide, although mostly asymptomatic [9]. HSV-2 infections are associated with oral, ocular, skin, genital, and central nervous system lesions and can result in mild or severe manifestations such as neonatal herpes, corneal blindness, meningitis, and encephalitis. Despite being commonly associated with genital herpes, epidemiological studies show that the presence of HSV-2 increases the susceptibility to acquisition, excretion, and transmission of other sexually transmitted infections [10-12] and is associated with an increased risk of developing invasive cervical carcinomas in HPV-seropositive women [11] and with the development of certain types of neoplasms.

Our group [4,5,13], among others, has been investigating the role of HSV infection in thyroid cancer development [14]. HSV is frequently found in thyroid nodules and the susceptibility to viral infection results from the increased expression of the viral entry mediator, nectin1, and the activation of mitogenic signaling [14]. *Nectin1* is a cell surface adhesion molecule that functions as a cell receptor for HSV, aiding viral entry through interaction with envelope glycoproteins, participating in the fusion process of the viral envelope in the cell plasma membrane [15-18]. In addition, *nectin1* plays an essential role in the formation of intercellular junctions, proliferation, differentiation, survival, and cell migration [19]. Recent studies have demonstrated that a high expression of nectin1 in malignant tumors is associated with tumor progression, worse prognosis, and greater susceptibility to herpesvirus infection [14,20,21]. In contrast, low expression of nectin1 has been suggested to inhibit the proliferation of tumor cells in cell culture, indicating a high potential of nectin1 as a therapeutic target [22]. Thus, the functional integrity of nectin1 seems essential for a tumorigenic role of HSV.

A large number of genes associated with various cancer types contain single nucleotide polymorphisms (SNPs). These SNPs may affect cancer susceptibility and may be critical in the molecular pathogenesis of various tumors. In addition, SNPs are potential diagnostic and therapeutic biomarkers in many cancer types, including thyroid neoplasms [23-28]. Many SNPs have been identified in the *NECTIN1* gene despite their functional role and clinical significance remaining mostly unclear.

The present study aimed to investigate a possible role of variants of *NECTIN1* gene on the susceptibility to herpesvirus and the development of thyroid cancer. We

used various computational tools to select missense SNPs with potential harmful impact on the protein structure and further investigated these SNPs in a large series of positive and negative HSV-2 thyroid nodule patients.

MATERIAL AND METHODS

In Silico Methods

Data mining

SNPs were identified from NCBI NP dbSNP (https://www.ncbi.gov/) using the keyword "*NECTIN1*". Of the 34,270 SNPs recovered, 548 non sinonimous (nsSNPs) were predicted to be probably pathogenic and selected for *in-silico* analysis. Interpretations of the pathogenicity of these nsSNPs in thyroid cancer have not been reported previously.

The reference sequence of *nectin1* was retrieved from Uniprot (ID: Q15223) and the raw data of *NECTIN1* gene were obtained from dbSNP database. Figure 1 shows the workflow of the *in-silico* tools used in the process of analysis and selection of SNPs.

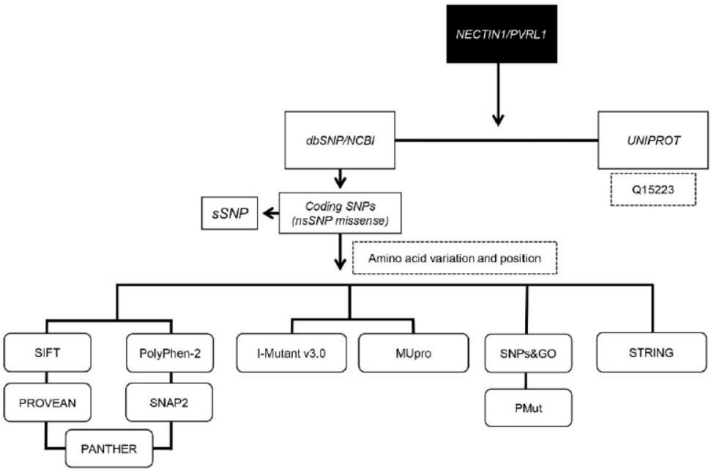

Figure 1: Flowchart of the *in-silico* prediction models employed.

In-silico Tools

Prediction and validation of nsSNPs pathogenicity

nsSNP are genetic mutations that cause a single amino acid substitution in the protein sequence, which can potentially influence protein function and structure, consequently changing its phenotype [29].

We used the *in-silico* analysis tools: SIFT, PolyPhen-2, PROVEAN, SNAP2, PANTHER, I-Mutant v3.0, MUpro, SNPs&GO, PMut, STRING for prediction and analysis of the pathogenicity of nsSNP in the NECTIN1 gene.

Prediction of the effect of protein variation

Two programs based on sequence homology: SIFT (classification of intolerants of tolerants) and PROVEAN (analyser of effects of protein variation) were used to predict the deleterious effect of 548 nsSNPs on protein function.

We employed SIFT (Sorting intolerant from tolerant) to assess the effect of a substitution on protein function. The tool predicts the important positions in the protein sequence that are conserved throughout evolution, therefore, those where important substitutions can affect protein function. The SIFT Score ranges from 0 to 1, and scores ≤0.05 are predicted by the algorithm as detrimental substitutions, while scores >0.05 are considered tolerable. The SIFT prediction process usually takes 5 minutes to 30 minutes to transform the amount of input data [30].

PROVEAN (Protein variation effect analyzer) (http://provean.jcvi.org) is an online prediction tool that estimates the effect of sequence variation on protein function. PROVEAN is based on clustering methodology that compares wild-type protein sequence and amino acid mutations. A variant is considered "deleterious" if the final score is below -2.5, and is considered "neutral" if the score is above -2.5 [31].

PolyPhen-2 (Polymorphism Phenotyping v2) uses physical and comparative approaches to provide accurate predictions about the effects of each amino acid substitution on the protein's structural and functional properties. It employs a series of sequence, phylogenetic, and structural features that characterize the substitution, providing three possible outcomes (probably harmful, possibly harmful, or benign) with score ranging from zero to 1.0 [32].

SNAP2 is a neural network-based functional tool that predicts the effects of single amino acid substitutions on protein function. SNAP2 makes its predictions considering a variety of structural features alongside the sequence of the wild-type protein and its variants. The prediction score ranges from -100 (for neutral prediction) to +100 (strong effect) which represents the probability of the mutation modifying the function of the native protein [33,34].

PANTHER predicts the probability of a given nsSNP to functionally impact a protein [35]. PANTHER estimates the replacement position-specific evolutionary conservation. The PSEL (Position specific evolutionary preservation) score employs as a measure the length of time (in millions of years) that a position is preserved in the protein.

Prediction of functional impact of mutations on proteins

Effects of nsSNPs on protein stability

I-Mutant v3.0 rates are based on protein sequence and are classified into three classes: neutral mutation (-0.5 ≤DDG ≥0.5 kcal/mol), large reduction (≤0.5 kcal/mol) and large increase (>0.5 kcal/mol). The free energy change (DDG) predicted by the tool is fundamental to the difference between the unfolding Gibbs free energy change of the native protein and the mutant protein (kcal/mol) [36].

MUpro is a web server for predicting protein stability changes after mutations. MUpro can predict changes in protein stability upon substitution of a single amino acid, simply using sequence information or combining this information with tertiary structure. The MUpro cutoff value of DDG is the same used in the I-Mutant [37].

Prediction of disease related mutations by SNPs&GO and PMut

The SNPs&GO algorithm is a web server that predicts the impact of protein mutations using information from the three main roots encoded by Genetic Ontology (GO) terms; molecular function, biological process, and cellular component [38]. From the FASTA sequence of the protein, SNPs&GO predicts the probability of the disease-related mutation effect on the function of the parent protein with a Matthews correlation coefficient of 0.63 and a precision of 82%.

PMut is a functional tool based on neural network (NNs) intelligence that allows you to accurately and quickly display pathological characteristics caused by a single amino acid substitution. The prediction can be considered neutral or disease-causing [39]. The input mechanism for PMut is the FASTA protein sequence or Swiss-Prot code. PMut also makes it possible to scan and display the location of the mutation in the protein structure, where available, using color coding to track the pathogenicity associated with the mutation. The pathogenicity result shows variation index from 0 to 1, where index >0.5 indicates pathological mutations [37].

Protein-protein interactions analysis STRING

Mutations can alter the function and structure of a protein; therefore, the mutated protein can interact with other proteins and show phenotypic effects. STRING server was used to investigate an interaction of nectin1 with other proteins. STRING is a web-based tool for gene retrieval and protein interaction; this usable resource aggregates available information about protein-protein association; scores predicted interactions and points out the results of automatic literature mining searches [40].

Validation Step

After the *in-silico* analysis and an evaluation of the linkage disequilibrium using the HaploView v4.2 software via HapMap (International HapMap Project), three *NECTIN1* polymorphisms considered deleterious were chosen to further investigate the genetic profile of thyroid benign and malignant nodules, and patients infected by HSV-2 and not, as shown in the supplementary files (Table 1).

Gene	Polymorphism	dbSNP code
NECTIN1	rs141253617	C_161185238_10
NECTIN1	rs199962982	C_189780609_10
NECTIN1	s7940667	C_1567672_10

Table 1: List of *NECTIN1* SNPs chosen for further real-time PCR validation in thyroid nodule patients.

Patients and controls

This retrospective part of the study was performed in accordance with the World Medical Association Code of Ethics for experiments involving human subjects. It was approved by the Research Ethics Committee of the Faculty of Medical Sciences (FCM) of UNICAMP under the protocol number #314791207.0000.5404. All included patients and control healthy individuals signed an informed consent form.

We examined 440 consecutive patients which were referred to the Clinical Hospital FCM/UNICAMP and the private clinic (TMB Surgical Clinic) Campinas/São Paulo for thyroid nodule investigation. All 440 patients, 72 men and 368 women aged 40.4 ± 10.3 years old on average, were submitted to fine needle aspiration to provide cytology samples that revealed that 71 (16.1%) had an adenomatous goiter; 40 (9.1%) had a follicular adenoma; 329 (56.4%) had a differentiated thyroid cancer including 25 papillary thyroid microcarcinomas (PTCM), 248 (5.7%) papillary thyroid carcinomas (PTC), and 25 (12.7%) follicular thyroid carcinomas (FTC).

Inclusion criteria were a clinical diagnosis of thyroid nodule and age between 18 years and 80 years. We excluded patients with previous thyroid tumors and pregnant women. Data on demographic characteristics, including age at diagnosis, gender, and occupation were obtained from medical records that also provided data on cytology and histology examination, patients' management, and follow-up.

In addition, 440 blood samples were obtained from healthy blood donors (137 men and 303 women, 38.9 ± 12.2 years old) recruited at the Center of Hematology and Hemotherapy of the University of Campinas, Brazil. These individuals had no history of thyroid disease, no sign on physical examination and/or no indication of thyroid abnormality on laboratory and/or image exams.

Polymorphism analysis

We collected peripheral venous blood from all 810 participant of this study. DNA extraction was performed with phenol and chloroform, precipitated, and stored at -20°C until use.

We employed the TaqMan® technique (Applied Biosystems™, Foster City, USA) for genotyping, using pre-engineered TaqMan probes purchased from Applied Biosystems (Thermo Fisher Scientific, Inc.). Table 2 shows the specific sequence of the amplicons.

The final volume of each PCR reaction was 7 μL comprised of 2 μL of DNA (>10 ng/μL), 3.5 μL of genotyping master mix (1 × final concentration), 0.175 μL of probe and primers (1 × final concentration), and 1.325

µL of sterile water. PCR conditions included the initial denaturation phase of 95°C for 10 minutes, followed by 40 cycles at 92°C for 15 seconds, then 60°C for 90 seconds. The PCR plates were read by the "Sequence Detection Software".

SNP	Context Sequence [VIC/FAM]
rs141253617	GCCTTGCTGTAGCCGTTGCCATACA**[C/T]**GTGCTTCTTGGTGCTGTAGTCACCC
rs199962982	TTGAGCTGGCTTTCTCGATTGCCCG**[C/T]**AGGGAAGGTAGCAAACTCGCAGATG
rs7940667	CAGGAAGAAGACAGTGAGCACAGCA**[A/C]**CTAGGATGAGGAACACGGCCACGGT

Table 2: Specific sequence of the analyzed amplicons.

Serological analysis by enzyme-linked immunosorbent assay (ELISA)

We obtained blood samples from 300 individuals out of the 810 participants of the investigation, including 150 thyroid nodule patients (21 men and 129 women, 42.34 ± 10.90 years old). There were 65 benign nodules (48 goiters, 17 AF) and 85 malignant nodules (29 MCPT and 56 CPT). Serology was also performed in 150 control individuals (21 men and 129 women, 45.0±11.0 years old).

We employed the commercial ELISA kit, HerpeSelect® 2 (Cat. No. EL0920G, Focus Diagnostics, USA) according to the manufacturers' instructions. The color intensity reading was measured in the ELx808™ spectrophotometer device (BIOTEK US) to read the optical density (OD) at 450 nm. The index value of each sample was obtained by dividing the OD by the mean absorbance of the kit controls. Samples with OD index value <0.90 were considered anti-HSV-2 negative, index values >1.10 were considered anti-HSV-2 positive, and index ranging between 0.90 and 1.10 were considered anti-HSV-2 indeterminate.

rsID	Amino acid	Prediction	Score	rsID	AA change	Prediction	Score
rs73571271	P395S	Damaging	0	rs267602722	E423K	Damaging	0.033
rs7940667	V361G	Damaging	0	rs367791177	N176S	Damaging	0
rs78809001	R199Q	Damaging	0.025	rs368140971	H409Q	Damaging	0.044
rs137991779	G44S	Damaging	0	rs368373192	V65L	Damaging	0.018
rs142930935	R212H	Damaging	0.007	rs368738833	N200D	Damaging	0.046
rs150553818	E330K	Damaging	0.01	rs369649445	A377G	Damaging	0.021
rs141253617	V395M	Damaging	0	rs370234311	T108A	Damaging	0
rs199962982	T131A	Damaging	0	rs370390168	R94H	Damaging	0.009
rs202095358	G507E	Damaging	0	rs371651972	T153A	Damaging	0.009
rs375956459	P340Q	Damaging	0	rs371752868	E475Q	Damaging	0.025
rs139388001	E406A	Damaging	0.029	rs372177799	R199W	Damaging	0.013
rs140089588	T206M	Damaging	0	rs373047519	R96H	Damaging	0
rs140974611	V265M	Damaging	0.008	rs374378792	Q76H	Damaging	0
rs143539245	N202S	Damaging	0.014	rs374562515	A144T	Damaging	0.01
rs147357554	I306N	Damaging	0.001	rs376335229	R380W	Damaging	0.008
rs185201594	R325C	Damaging	0.004	rs377427305	T131M	Damaging	0.007
rs199772536	L18F	Damaging	0.034				

Table 4: SIFT prediction analysis of 33 *NECTIN-1* nsSNPs.

Statistical analysis

Data analysis was performed with the GraphPad Prism software (©2018 GraphPad Software, Inc, USA). Categorical variables were presented with values of absolute frequency (n) and percentage (%), and parametric continuous variables were presented as the mean ± SD (standard deviation) and were compared using the unpaired Student's t-test. Clinical and pathological differences between thyroid nodule patients and controls were

evaluated using the $\chi 2$ test, and, when necessary, Fisher's exact test. The 95% confidence interval (CI) was adopted in all tests and a p-value <0.05 was considered to indicate a statistically significant difference.

RESULTS

In-Silico

We evaluated all 548 nsSNPs and the *NECTIN1* genes retrieved from the dbSNP using state-of-the-art bioinformatics tools.

nsSNPs found by SIFT

We used the rsID retrieved in dbSNP as input to the SIFT server and Table 4 shows the results classified as deleterious. The lower the tolerance index (score), the greater the functional impact that an amino acid residue substitution is likely to have, and vice versa. Out of the 548 nsSNPs analyzed, we identified 33 nsSNPs as deleterious with a tolerance index ≤0.05.

	PolyPhen-2		PROVEAN		SNAP2			PANTHER	
AA change	**Prediction**	**Score**	**Prediction**	**Score**	**Prediction**	**Score**	**Preservation Time**	**Prediction**	**Pdel**
P395S	-	-	-	-	-	-	-	-	-
V361G	Damaging	0.799	-	-	-	-	-	-	-
R199Q	Damaging	1.000	Neutral	-0.496	Disease	0.460	361	possibly damaging	0.5
G44S	-	-	Damaging	-4.724	Disease	0.630	842	possibly damaging	0.78
R212H	Damaging	1.000	-	-	Disease	0.505	361	possibly damaging	0.5
E330K	Damaging	0.979	Neutral	-1.239	Disease	0.655	362	possibly damaging	0.5
V395M	Damaging	0.997	Neutral	-0.858	Neutral	0.390	362	possibly damaging	0.5
T131A	BENIGN	0.002	Neutral	-0.330	Neutral	0.290	361	possibly damaging	0.5
G507E	-	-	Damaging	-4.027	Neutral	0.500	56	possibly damaging	0.57
P340Q	BENIGN	0.003	Neutral	-0.123	Disease	0.650	456	possibly damaging	0.57
E406A	-	-	-	-	-	-	-	-	-
T206M	Damaging	1.000	Damaging	-5.448	Disease	0.685	750	possibly damaging	0.74
V265M	Damaging	0.999	Neutral	-2.451	Disease	0.610	362	possibly damaging	0.5
N202S	Damaging	0.999	Damaging	-3.170			362	possibly damaging	0.5
I306N	Damaging	0.999	Damaging	-4.523	Disease	0.520	176	probably benign	0.27
R325C	Damaging	1.000	Damaging		Neutral	0.445	362	possibly damaging	0.5
L18F	BENIGN	0.999	Neutral	-5.313	Disease	0.655	220	possibly damaging	0.5
E423K	-	-	-	-	-	-	-	-	-
N176S	Damaging	1.000	Neutral	-1.851	Neutral	0.485	456	possibly damaging	0.57
H409Q	Damaging	0.917	Neutral	-0.326	Disease	0.540	362	possibly damaging	0.5
V65L	BENIGN	0.259	Neutral	-1.6	Neutral	0.340	176	probably benign	0.27
N200D	Damaging	0.985	Neutral	-1.213	Disease	0.560	176	probably benign	0.27
A377G	BENIGN	0.018	Neutral	-1.817	Neutral	0.280	176	probably benign	0.27
T108A	Damaging	1.000	Damaging	-3.881	Disease	0.675	456	possibly damaging	0.57
R94H	Damaging	0.995	Neutral	-1.768	Neutral	0.255	176	probably benign	0.27
T153A	Damaging	0.729	Neutral	-1.315	Neutral	0.395	362	possibly damaging	0.5
E475Q	Damaging	0.993	Neutral	-0.743	Neutral	0.220	176	probably benign	0.27
R199W	Damaging	1.000	Damaging	-3.296	Disease	0.695	361	possibly damaging	0.5
R96H	Damaging	1.000	Damaging	-4.314	Neutral	0.450	750	possibly damaging	0.74
Q76H	Damaging	1.000	Damaging	-3.162	Neutral	0.430	456	possibly damaging	0.57
A144T	Damaging	0.998	Neutral	-1.87	Disease	0.550	456	possibly damaging	0.57
R380W	Damaging	1.000	Damaging	-4.008	Disease	0.695	324	possibly damaging	0.5
T131M	Damaging	0.998	Neutral	-0.345	Neutral	0.230	361	possibly damaging	0.5

(-) does not match the sequence residue

Table 5: Results from nsSNP Analyzer, PolyPhen-2, PROVEAN, SNAP2 and PANTHER.

Protein variation effect analysis by PolyPhen-2 and PROVEAN

We analyzed the effect of the variant on the biological function of the protein, based on sequence homology, with PolyPhen-2 and PROVEAN. PolyPhen-2 classified 23 out of 33 polymorphisms as highly deleterious, 5 as benign, and 5 presented residue sequences that did not match. The PROVEAN tool, out of the 33 nsSNPs, 11 were considered "deleterious" and 17 were considered "neutral", did not identify the sequence of residues in 5 variants. SNAP2 predicted 16 nsSNPs as deleterious and their scores are

shown to have the effect of causing the phenotype in diseases (Table 5).

Protein variation effect analysis by PANTHER

We used the FASTA protein sequence as input for evolutionary analysis of coding SNPs and analysis of deleterious effects on protein function. The probability of deleterious effect (Pdel) value is a qualitative conversion where the time "<200 my is probably benign", the time ">200 my is possibly harmful" and the time ">450 my is probably harmful". Of the 33 deleterious nsSNPs, 9 were considered likely to damage the functionality (Table 3).

Impact of gene variations on corresponding proteins

We evaluated the impact on the structural stability of nectin1 by I-Mutant v3.0 using a standard free energy change at pH 7.0 and temperature of 25°C as a basis. A predicted free energy change (DDG) value of >0 means an increase in protein stability and a DDG <0 means a decrease in protein stability. Out of the 33 nsSNPs, I-Mutant v3.0 identified 28 as reducing stability whereas 5 variants increased it Table 5. We also analyzed variants with decreased and increased protein stability regarding their predicted pathogenicity, Twelve of 33 nsSNPs deleterious disease-related mutations; 06 in SNPs&GO and 6 in PMut (Table 5).

To understand the interactions of nectin1 with other proteins, we performed interaction analysis using the STRING tool. A network of 51 proteins were recognized to bind nectin1 in the STRING database. The resulting network shows three evident clusters in which nectin1 is connected to at least ten other proteins with the most reliable binding (Figure 1). A first cluster consisted of 8 proteins identified with virus receptor activity and cell adhesion and (PAEP, CD96, MLLT4, PVRL3, PVRL4, ACKR1 and TNFRF14) (Figure 1A). A second cluster consisted of 39 proteins associated mainly with cell adhesion and transcription factors, of which 5 of them (CTNNB1, TCF7, TCF7L1, LEF1 and CDH1) were previously studied in thyroid cancer (Figure 1B). And a third cluster identified 4 proteins (KLRG1, CD226, PVR and BTLA) associated in the immunological process and as a mediator of herpesvirus entry (Figure 1C). As predicted, the nectin1 interaction network is integrated with numerous proteins that are tightly regulated in cell adhesion and virus receptor, including BTLA, PVR, PVRL3 and PVRL4.

Analysis of minor allele frequency and linkage disequilibrium

Not all SNPs in dbSNP are real, some may have arisen exclusively due to sequencing errors or may be unique to certain individuals, which may justify not finding the sequence in various tools. Thus, we used additional resources to verify the results reliability [41]. To prioritize the selection of functional SNPs, we also used the lowest allele frequency (MAF) and linkage disequilibrium (LD) analysis, since MAF represents the sample size to detect variant alleles of an SNP in a given population and is linked to the statistical power of the study [42] and linkage disequilibrium is the non-random association of alleles at two or more loci in a population and is directly related to a population's history of mutation and recombination [43]. Therefore, most large-scale genome studies generally target SNPs with a MAF of 0.05 or more and seek weak linkage disequilibrium as an ideal condition. With all this in mind and based on the bioinformatics results, we selected SNPs with MAF ≥0.05 and with weak LD. We evaluated the LD using the HaploView v4.2 program and recorded the MAF values based on the dbSNP database. Figure 2 shows these results.

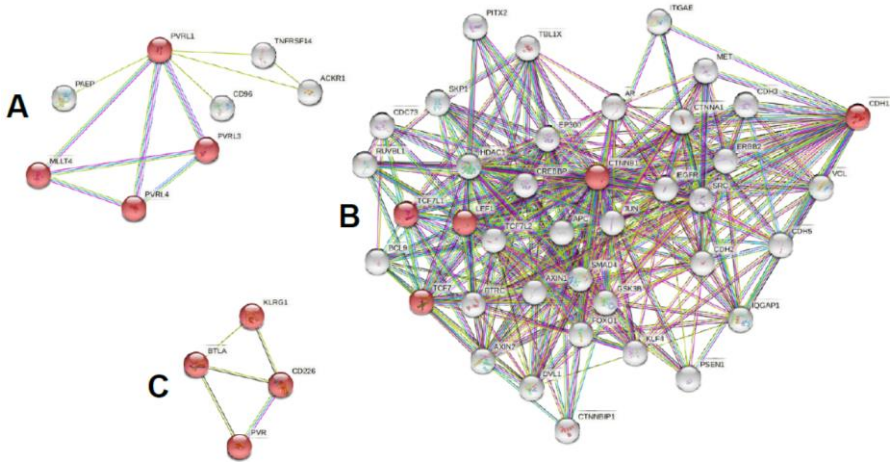

Figure 1: Nectin-1 interacts with other proteins according to STRING network graphic.

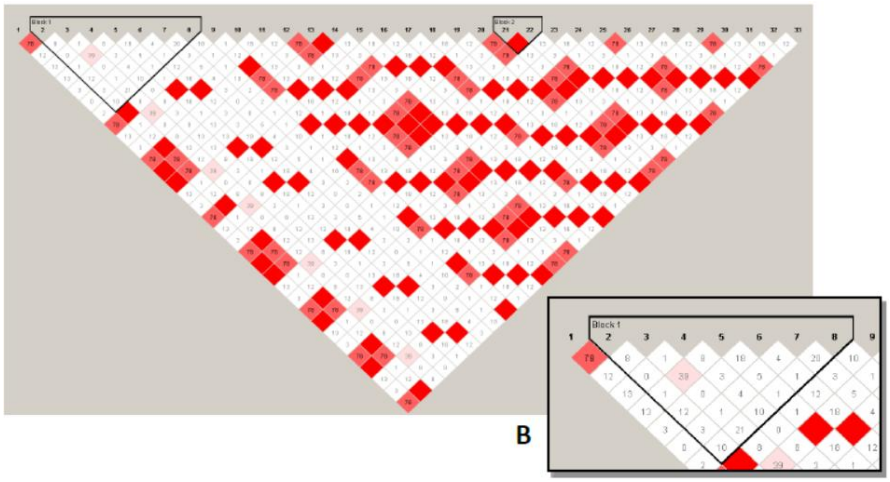

Figure 2: Schematic representation of haplotype block. Panel A shows all SNPs and Panel B shows the haplotype block of SNPs with weak and moderate linkage disequilibrium.

As shown in Figure 4 haplotype pairs from SNP 2 to 8 show no significant LD. The highest LD was 39 between rs78809001 and rs142930935 of the *NECTIN1 gene*, indicating a moderate linkage disequilibrium.

In summary, only 3 out of the 33 deleterious *NECTIN1* SNPs were considered functionally important and selected for validation studies. Of these three SNPs, only rs7940667 presented MAF ≥0.05. Despite the MAF<0.05 for rs141253617 and the weak LD of rs199962982, we included both these SNPs in the study. Other SNPs with MAF <0.05 or not validated (Table 6) were excluded from further analysis.

Seroprevalence of HSV-2

We observed HSV-2 seropositivity in 37 (25%) of the thyroid nodule patients and 42 (28%) of the controls (p = 0.5122). The mean age of seropositive individuals was higher (47.4 ± 10.0 years old) than that of HSV-2 negative individuals (41.2 ± 10.9; p = 0.0001; OR = 2.681, 95%, CI = 1.434 - 9.483). Seropositivity rate was similar in benign (49%) and malignant (51%; p = 0.4522) thyroid nodule patients and between women (84%) and men (16%; p = 0.3415). The seroprevalence for HSV-2 was not associated with any clinical or pathological characteristic of thyroid nodules (Table 7).

	I-Mutant v3.0		MUpro		SNPs&GO			PMut	
AA change	**Prediction**	**DDG (kcal/mol)**	**Prediction**	**Score**	**Prediction**	**RI**	**Probability**	**Score**	**Prediction**
P395S	Decrease	-1.88	decrease stability	-1.88	-	-	-	-	-
V361G	Decrease	-2.03	decrease stability	-2.03	-	-	-	-	-
R199Q	Increase	0.53	Increases stability	0.53	Neutral	9	0.071	0.26	Neutral
G44S	Decrease	-1.92	decrease stability	-1.92	Disease	7	0.84	0.66	Disease
R212H	Decrease	-1.29	decrease stability	-1.29	Neutral	6	0.213	0.17	Neutral
E330K	Decrease	-2.38	decrease stability	-2.38	Neutral	2	0.411	0.27	Neutral
V395M	Decrease	-1.46	decrease stability	-1.46	Neutral	9	0.058	0.08	Neutral
T131A	Decrease	-0.78	decrease stability	-0.78	Neutral	9	0.028	0.04	Neutral
G507E	Increase	0.22	Increases stability	0.22	Disease	0	0.51	0.39	Neutral
P340Q	Decrease	-0.8	decrease stability	-0.8	Neutral	8	0.084	0.15	Neutral
E406A	Decrease	-1.4	decrease stability	-1.4	-	-	-	-	-
T206M	Decrease	-1.28	decrease stability	-1.28	Disease	1	0.559	0.55	Disease
V265M	Decrease	-1.43	decrease stability	-1.43	Neutral	6	0.184	0.45	Neutral
N202S		-0.53	decrease stability	-0.53	Neutral	6	0.21	0.12	Disease
I306N	Decrease	-1.61	decrease stability	-1.61	Neutral	1	0.442	0.54	Neutral
R325C	Decrease	1.98	decrease stability	1.98	Disease	0	0.518	0.58	Disease
L18F	Increase	0.48	decrease stability	0.48	Neutral	8	0.116	0.14	Disease
E423K	Increase	-0.93	decrease stability	-0.93	-	-	-	-	Neutral
N176S		-1.29	decrease stability	-1.29	Neutral	6	0.183	0.38	-
H409Q	Decrease	-1.89	decrease stability	-1.89	Neutral	6	0.186	0.13	-
V65L	Decrease	-0.56	decrease stability	-0.56	Neutral	8	0.079	0.15	Neutral
N200D	Decrease	0.11	Increases stability	0.11	Neutral	7	0.139	0.14	Neutral
A377G	Decrease	-1.82	decrease stability	-1.82	Neutral	7	0.147	0.14	Neutral
T108A	Increase	-0.87	decrease stability	-0.87	Neutral	6	0.204	0.37	Neutral
R94H	Decrease	-0.34	decrease stability	-0.34	Neutral	7	0.148	0.13	Neutral
T153A	Decrease	-2.24	decrease stability	-2.24	Neutral	9	0.071	0.13	Neutral
E475Q	Decrease	-0.51	decrease stability	-0.51	Neutral	6	0.186	0.12	Neutral
R199W	Decrease	0.2	Increases stability	0.2	Neutral	7	0.141	0.55	Neutral
R96H	Decrease	-0.24	decrease stability	-0.24	Neutral	6	0.19	0.52	Neutral
Q76H	Increase	-1.14	decrease stability	-1.14	Neutral	9	0.071	0.3	Disease
A144T	Decrease	-0.89	decrease stability	-0.89	Disease	0	0.504	0.33	Disease
R380W	Decrease	1.37	Increases stability	1.37	Disease	4	0.72	0.44	Neutral
T131M	Decrease	-0.27	decrease stability	-0.27	Neutral	9	0.071	0.15	Neutral

(-) does not match the sequence residue

Table 6: Total results and predictions by all 4-mutation predicting in silico tools. Tools are: I-Mutant v3.0, MUpro, SNPs&GO and PMut.

nº	**rsID**	**MAF**	**nº**	**rsID**	**MAF**
1	rs73571271	<0.01	**18**	rs267602722	ND
2	rs7940667	0.13	**19**	rs367791177	<0.01
3	rs78809001	<0.01	**20**	rs368140971	<0.01
4	rs137991779	<0.01	**21**	rs368373192	<0.01
5	rs142930935	<0.01	**22**	rs368738833	<0.01
6	rs150553818	<0.01	**23**	rs369649445	<0.01
7	rs141253617	0.01	**24**	rs370234311	ND
8	rs199962982	0.01	**25**	rs370390168	<0.01
9	rs202095358	<0.01	**26**	rs371651972	<0.01
10	rs375956459	<0.01	**27**	rs371752868	ND
11	rs139388001	<0.01	**28**	rs372177799	<0.01
12	rs140089588	<0.01	**29**	rs373047519	<0.01
13	rs140974611	<0.01	**30**	rs374378792	<0.01
14	rs143539245	<0.01	**31**	rs374562515	<0.01
15	rs147357554	<0.01	**32**	rs376335229	<0.01
16	rs185201594	<0.01	**33**	rs377427305	<0.01
17	rs199772536	<0.01			

Table 7: MAF frequency list and ratio of SNPs analyzed for linkage disequilibrium.

Genotyping investigation rs7940667

The genotypic distribution of rs7940667 polymorphism was similar in patients (A/A = 50.4%; A/C+ C/C = 48.1%) and controls (A/A = 49.6%; A/C+C/C = 51.9%; p = 0.0.6000). The genotypic frequencies were also similar in benign (A/A = 25%; A/C+C/C = 20.0%) and malignant thyroid nodules (A/A = 75%; A/C+C/C = 80%; p =

0.4319). Table 8 shows that there was no correlation between any clinical or pathological characteristic of the patients and the genotype profile of NECTIN1 rs7940667, except for capsular invasion, which was less frequent in patients with rs7940667 variants (p = 0.0105). In fact, the presence of these variants more than doubled the protection against capsular invasion (OR = 2.232, 95%CI = 1.150-4.380).

In the univariate analysis, we did not observe differences between the positives for HSV-2 in the presence of the genotype (A/C+C/C) rs7940667 (p = 0.2147). Although we observed that serological anti HSV-2 IgG titers were higher in patients with thyroid nodule with genotype A/C+C/C. With IgG titers of 1.4 ± 3.0 IU/mL in A/A and 2.6 ± 4.2 IU/mL in A/C+C/C (p = 0.0950). Only two thyroid nodule patients - an adenomatous goiter and a papillary microcarcinoma, both HSV-2 negative - showed the C/C allele of rs7940667.

rs199962982 and rs141253617

rs199962982 and rs141253617 did not show variation in genotype: All patients and controls were wild type homozygous.

	Genotypes			
Clinical and pathological features n (%)	**rs7940667 AA (N = 720)**	**rs7940667 AC+CC (N = 160)**	**Total (N = 880)**	**p value**
Tumor size (cm) (mean ± SD)	1.7 ± 1.0 (N=301)	1.8 ± 1.3 (N=65)	1.7 ± 1.1 (N=366)	0.8583[#]
Title Antibody (Mean ± SD (N))	1.4 ± 3.0 (N=249)	2.6 ± 4.2 (N=51)	1.6 ± 3.2 (N=300)	0.0950[#]
Group				
Case	363 (50.4%)	77 (48.1%)	440 (50.0%)	0.6000[##]
Control	357 (49.6%)	83 (51.9%)	440 (50.0%)	
Gender				
Women	546 (75.8%)	125 (78.1%)	671 (76.3%)	0.5378[##]
Men	174 (24.2%)	35 (21.9%)	209 (23.8%)	
Nodule				
Benign	84 (24.6%)	15 (20.3%)	99 (23.8%)	0.4319[##]
Malignant	258 (75.4%)	59 (79.7%)	317 (76.2%)	
Histology				
Adenoma	27 (7.9%)	5 (6.8%)	32 (7.7%)	0.8488[##]
Goiter	57 (16.7%)	10 (13.5%)	67 (16.1%)	
Microcarcinoma	42 (12.3%)	7 (9.5%)	49 (11.8%)	
Papillary	196 (57.3%)	47 (63.5%)	243 (58.4%)	
Follicular	20 (5.8%)	5 (6.8%)	25 (6.0%)	
Capsular invasion				
Absent	96 (53.3%)	42 (72.4%)	138 (58.0%)	0.0105[##]
Present	84 (46.7%)	16 (27.6%)	100 (42.0%)	
Vascular invasion				
Absent	57 (77.0%)	30 (88.2%)	87 (80.6%)	0.1717[##]
Present	17 (23.0%)	4 (11.8%)	21 (19.4%)	
TCL				
Absent	110 (82.7%)	24 (82.8%)	134 (82.7%)	0.9947[##]
Present	23 (17.3%)	5 (17.2%)	28 (17.3%)	
Multifocality				
Absent	128 (55.4%)	29 (60.4%)	157 (56.3%)	0.5247[##]
Present	103 (44.6%)	19 (39.6%)	122 (43.7%)	

TCL: Chronic Lymphocytic Thyroiditis. [#]Mann-Whitney test; [##]Chi-square test

Table 8: Genotypic distribution of the rs7940667 polymorphism according to thyroid nodule patients' clinical and pathological characteristics.

DISCUSSION

Nectins are cell-surface proteins that, apart from mediating cell-cell adhesion, are also involved in multiple signaling pathways related to cell proliferation, morphogenesis, growth, development, and immune modulation [44]. *Nectin-1* is normally expressed in various epithelial tissues and has been shown to serve as a receptor for viral entry [45]. A reduction in nectin-1 expression is observed in tumors of epithelial origin and at the edges of migrating cells during the early stages of malignant transformation, suggesting a role in reducing cell-cell adhesion and increasing invasiveness and metastasis [46].

The influence of *NECTIN1* gene variants on the function and structure of nectin1 protein and their role in thyroid nodule malignancy associated to HSV-2 infection have not been previously investigated. In order to identify and select potentially harmful nsSNPs in the *NECTIN1* gene, we performed in silico analyzes utilizing a series of bioinformatics tools designed to predict the biological consequences of these polymorphisms. We selected three nsSNPs predicted to damage nectin1 morphology and function that were further validated in a relatively large cohort of thyroid nodule patients and healthy controls. Despite careful selection, only rs7940667, but not rs199962982 and rs141253617, showed genotypic variation in our population.

Although the genotypic profile presented similar distribution in thyroid nodules and in the general population, we observed that the C/C genotype of rs7940667 occurred in only two thyroid nodule patients, both HSV-2 negative, which could suggest that this variant impairs the interaction of *nectin-1* with gD glycoprotein. In fact, our *in-silico* analysis demonstrated that the exchange of glycine for a valine at position 361 results in reduced stability of the protein, functional and structural changes in the transmembrane region of *nectin-1* [47]. These changes reduce the efficiency of cell binding, impairing the cell fusion process and the entry of the herpesvirus [48].

We also observed that patients with altered genotype (AC+CC) had tumors that presented less frequently capsular invasion at diagnosis, suggesting that the variant genotype could be a protective factor against aggressive phenotypes of thyroid cancer. Although PTC usually follows an indolent clinical course and has an extremely low long-term disease-specific mortality rate, it is still highly metastatic and recurrent [49].

Extrathyroidal extension is recognized as a significant prognostic factor since the probability of aggressive behavior increases proportionally to the extent of invasion [50,51]. Nectins have a key role in cell adhesion and, therefore, in cell-to-cell communication. The loss of this junction, either because of extracellular injury or neoplastic transformation, leads to a breakdown of cellular integrity with loss of cellular architecture, mainly in invasive carcinomas [44]. Our data suggest that *Nectin-1* rs7940667 variant may indicate less aggressiveness and, if our observation is confirmed in a larger cohort, it may function as a useful prognostic clinical tool.

To the best of our knowledge, this is the first study looking at rs7940667 polymorphism in thyroid cancer. Unfortunately, our study has several limitations. The seroprevalence of anti-HSV-2 antibodies in our cohort was higher than that previously described in Brazil [52], but similar in patients with thyroid nodule and controls, neither distinguishing malignant from benign nodules, nor having an association with *Nectin-1* genotypic profile. A possible protective function of the *NECTIN1* variants and their influence on the role of herpes virus in thyroid carcinogenesis and tumor aggressive features need to be confirmed in larger cohorts, different populations and by functional studies.

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