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The Investigation of *WRAP53* rs2287499 Association with Thyroid Cancer Risk and Prognosis among the Azeri Population in Northwest Iran

Esmaeil Darvish Aminabad, M.Sc.[1], Aieda Sedaie Bonab, M.Sc. [2], Mohammad Ali Hosseinpour Feizi, Ph.D. [3], Nasser Pouladi, Ph.D. [4], Reyhaneh Ravanbakhsh Gavgani, Ph.D. [5]

1- Department of Biological Sciences, Faculty of Natural Siences, Ahar Branch, Islamic Azad University, Ahar-Iran

2- Department of Biology, Faculty of Natural Sciences, Tabriz University, Tabriz-Iran

3- Professor, Department of Animal Biology, Faculty of Natural Sciences, Tabriz University, Tabriz, Iran (Corresponding author; pourfeizi@eastp.ir)

4- Assistant Professor, Department of Cellular and Molecular Biology, Faculty of Science, Azarbaijan Shahid Madani University, Tabriz-Iran

5- Department of Animal Biology, Faculty of Natural Science, University of Tabriz, Tabriz, Iran

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Abstract

Background: *TP53* and the oncogene *WRAP53* are adjoining genes, producing p53-WRAP*53α* sense-antisense RNA couples. WRAP53α is indispensable for p53 mRNA regulation and p53 induction following DNA damage. Up-regulated WRAP53β can induce neoplastic transformation and cancer cell survival. All these, along with the associations of *WRAP53* single nucleotide polymorphisms with tumor incidence and prognosis, highlighted an impact in human cancers. Considering the importance of *WRAP53* in modulating p53, and the frequent occurrence of thyroid cancer, we examined the association of a *WRAP53* SNP (rs2287499) with thyroid cancer risk and prognosis among Iranian-Azeri population.

Methods: This research was done in Tabriz-IRAN in 2014. DNA samples obtained from 106 patients and 196 controls were subjected to polymerase chain-reaction-based single-strand conformational polymorphism (PCR-SSCP) analysis. Genotypes were characterized by sequencing. Correlations of desired SNP with thyroid cancer as well as age, gender, involved thyroid lobe, lymph node metastasis, tumor type, stage, and size were estimated using Chi-square (χ^2) or Fisher's exact tests with a *P*-value less than 0.05 as significant.

Results: rs2287499 is not associated with thyroid cancer predisposition. Except for gender, none of the clinicopathologic factors were significantly linked to the examined genotypes.

Conclusions: rs2287499 is not a genetic risk factor for thyroid cancer. Although rs2287499 is not assessable as a biomarker to predict prognosis based on clinicopathologic parameters, the considerable association with gender suggests that this SNP may indirectly be relevant to gender-associated disease manifestation. Further investigations on distinct types of thyroid tumors are needed to fully characterize the rs2287499 status in thyroid malignancies.

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Introduction

Throughout the world, the highest proportion of endocrine malignancies belongs to thyroid cancer, accounting for almost 3.5% and 0.9% of all cancers in females and males respectively. The incidence is generally higher in females than in males, with a worse prognosis mostly among men. In the

recent 5 years, there has been an approximate global increase of 1.6% in cases (1). In IRAN, thyroid gland neoplasia is the 11th most common cancer in both sexes, comprising 76.1% of endocrine and 1.8% of all diagnosed cancers (2, 3). As GLOBOCAN (Global Burden of Cancer Study) reported, the highest number of Iranian thyroid cancer patients arebetween 15-39 years of age (1). Considering the given statistics, we are facing a worldwide threat. For this reason, identification of thyroid cancer susceptibility loci is quite crucial to discovering stronger screening and prognostic biomarkers as well as avenues for therapeutic interventions. Thyroid tumorigenesis can originate from follicular epithelium, para-follicular (C-cells) and non-epithelial stromal components (4). The most prevalent types are epithelial tumors that derive from follicular cells and are categorized into 3 main groups: 1- benign (follicular adenomas), 2- malignant (follicular, anaplastic, well-differentiated papillary and poorly differentiated carcinomas), and 3- hyalinizing trabecular tumors with uncertain malignant potential. Medullary thyroid carcinoma is another form that arises from para-follicular cells (5). Several factors such as age, gender, radiation exposure, hormones ,reproduction, dietary iodine, family history, goiter, and other benign thyroid conditions have been implicated in thyroid cancer etiology(6). Besides, a number of hereditary factors and gene abnormalities have been proposed as potential risk factors (7-12).

TP53 (Tumor Protein 53) (17p13.1), which encodes p53 as an inevitable tumor suppressor, has been prevalently correlated to cancer predisposition. To properly monitor genome integrity, p53 undergoes strict regulations at both post-transcriptional and post-translational levels (13). A regulation by means of "Antisense Transcription" occurs in bi-directionally transcribed mammalian genes. In this system, transcription from the antisense strand of a gene produces an antisense transcript that regulates stability, transport, and translation of the matched sense transcript (14, 15). *WRAP53* (WD40-encoding RNA Antisense to p53) (17p13.1), considered as a candidate thyroid cancer susceptibility gene, is located on the antisense strand to *TP53* and partly overlaps it in a head-to-head orientation. The conservation of the close location of *WRAP53-TP53* in mammals during evolution is indicative of a biological functional significance. *WRAP53* is universally expressed in tissues and contains 13 exons with 3 alternative start exons (1α, 1β & 1γ) (16). The complementary sequence to the 5′ UTR of p53 mRNA is a highly conserved region, existing only in α-containing transcripts (WRAP53α) (17). The 1st exon of p53 directly overlaps the *WRAP53* exon 1α by up to 227 base pairs. *WRAP53* is transcribed in an opposite orientation relative to *TP53*, forming a p53-WRAP53α sense-antisense pair. Subsequent to WRAP53α/p53 RNA duplex formation, WRAP53α regulates and maintains basal p53 mRNA levels by protecting it from degradation. *WRAP53* expression takes place coincidentally with *TP53* expression upon DNA damage, indicating the pivotal role of WRAP53α in stabilizing p53 mRNA, and inducing p53 protein for preventing cells from becoming cancerous (16). However ,it is not clear whether the molecular mechanism of enhanced p53 stability is linked to the more stable conformation of RNA hybrids or the prevention of destabilizing factors from binding p53 mRNA (17). Nevertheless, it is evident that WRAP53α increases p53 mRNA and protein levels when overexpressed. Likewise, ectopic expression of this transcript potentiates p53-mediated apoptosis. In spite of this, WRAP53α depletion or WRAP53α/p53 hybrid blockage significantly reduces p53 induction and target gene transactivation (16, 17). Notably, WRAP53α regulates both wild-type and mutant p53 levels, and therefore,*WRAP53* can be targeted as a therapeutic strategy for mutant p53-carrying tumors (16). In contrast to WRAP53α,whichacts in favor of p53, WRAP53β encodes a

protein that exhibits oncogenic functions. WRAP53β was found to be overexpressed in a large spectrum of human cancer cell lines, resultingin cellular transformation and survival. However, its silencing ended up with cancer cell apoptosis (18). Moreover, WRAP53β participates in telomerase holoenzyme complex as an essential element for elongating telomeres in cancer cells where this enzyme is unexpectedly activated (19, 20).

Taking into account the critical role of *WRAP53* in p53-dependent biological response to DNA damage, WRAP53*α* malfunction could result in failure to sustain p53 expression, which finally contributes to cancer initiation or progression. It isalso quite important to study the potential *WRAP53* SNPs that may alter p53 efficacy, leading to tumorigenesis and poor prognosis. Consistently, a number of *WRAP53* alternations and SNPs have been associated with cancer risk and prognosis, strengthening the involvement of this gene in the pathogenesis of human malignancies (21-27). The present work is the first study evaluating a common *WRAP53* missense SNP (rs2287499 [Ex1, C >G, R68G]) in relation to thyroid cancer risk as well as prognosis in the Iranian-Azeri population. Here, we aimed to figure out whether this variation makes a person susceptible to develop thyroid tumor. The second objective was to determine the prognostic value of rs2287499.

Materials and Methods

Study population

This case-control association study was conducted in 2014 in Tabriz, IRAN. Subjects are all selected from the Azeri population of Northwest IRAN, including 106 thyroid cancer patients (81 females- 25 males) with the median diagnosis age of 37.5 (14-81 years), as well as 196 healthy controls (165 females- 31 males) with no cancer history in first- and second-degree family members. Cases were affected by different types of thyroid cancer .However, regardless of sex, most cases were in the 29-44 age group when diagnosed with this malignancy. All patients had undergone thyroidectomy at Noor-E-Nejat or Imam Reza hospital of Tabriz-IRAN between 2010 and 2013.

Sample collection and genomic DNA extraction

The peripheral blood samples from volunteer healthy donors were gathered at the Biology department of Tabriz University. After surgical resections, peripheral blood and tumor tissue samples as well as patient's medical records were obtained with informed consent. Tumor staging was then established according to the clinicopathological data and AJCC (American Joint Committee on Cancer) Tumor-Node-Metastasis staging system (28). Extraction of DNA was done on all subjects'peripheral blood samples using SDS/proteinase K and salting-out method (29). The quality and quantity of extracted genomes were determined by a Picodrop™ spectrophotometer (Bioneer Inc., Korea), and DNA samples with plausible absorbance ratios were subsequently frozen at -20ºC.

PCR-SSCP reactions

To analyze DNA samples for *WRAP53* codon 68 polymorphism, a pair of forward 5'-GGTTGTCCCCAGATCCTGT-3' and reverse 5'-ACTCTGTTTCCAGGGGAGTG-3' site-specific primers were used to amplify a target sequence (93bp) containing the desired polymorphic region (30). Each PCR reaction was conducted in a total volume 25µl reaction mixture prepared from1-2µl DNA template (20-50ng), 0.5µl dNTPs (10mM), 1µl of each primers (10pmol), 0.75µl $Mgcl_2$ (50mM), 2.5µl PCR buffer (10X), 18.05µl sterile distilled water, and 0.2µl Taq DNA polymerase (5U/µl) (Cinnagen, IRAN). In a thermal cycler (Sensoquest, GmbH, Germany), a PCR cycling

program was set as follows: an initial denaturation step (600sec–95ºC) followed by 35 cycles of denaturation (30sec–95ºC), primer annealing (30sec–59ºC), polymerisation (30sec–72ºC), and a final extension (600sec–72ºC). After amplification reaction, 4µl of individual PCR amplicons were mixed with 12µl of denaturing loading dye (95% formamide, 10mM NaOH, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). The mixtures were heat-denatured (600sec–96ºC) in a thermal cycler, and then snap-chilled on ice to stabilize single-stranded DNA amplicons. Denatured fragments were immediately loaded onto a polyacrylamide gel (22%), comprised of 5ml acrylamide-bisacrylamide solution (40%), 13.5ml deionized-distilled water, 3.5ml Tris-Borate-EDTA buffer (5X), 300µl ammonium persulphate (10%), and 30µl tetramethylenediamine. A DNA molecular size marker (50bp) (Fermentas, USA) was also loaded into a well. Using a vertical electrophoretic apparatus and a power supplier (Apelex, France), electrophoresis was performed in TBE buffer (0.6X) at 4°C and 100V/cm for 8-9h.

Visualization and SNP genotyping

After adequate separation of conformers, silver nitrate staining was done to visualize the distinct SSCP bands (31, 32). As expected, three different patterns were found to be associated withdifferent genotypes. To confirm the genotypes, three samples from each of the different patterns were then characterized through the Sanger sequencing method (Applied Biosystems 3730/3730xl DNA analyzers, Bioneer, Korea), which linked each banding pattern to a specific *WRAP53* rs2287499 genotype. Accordance of sequencing outcome with the original *WRAP53* sequence from the database NCBI (The National Center for Biotechnology Information) (Reference Sequence: NC.000017.11) was checked by Chromas software (v.2.4; Technelysium Pty Ltd., Australia).

Statistical analysis

SPSS (Statistical Package for the Social Sciences) (v.16; SPSS Inc., USA) and the Javastat online statistics packages (http://statpages.org/ctab2x2) were used for calculations and comparisons. Hardy-Weinberg equilibrium was tested for rs2287499 in both groups by using an online calculator (33). To evaluate the relation between rs2287499 and thyroid cancer risk as well as prognosis, the differences in allele and genotype frequencies between study groups and the rs2287499 association with clinicopathological features were assessed through Pearson's chi-square or Fisher's exact tests. *P* values and odds ratios (OR) with 95% confidence interval (CI) were calculated per allele and genotype in relation to thyroid cancer risk. The results were considered to be statistically significant provided that the *P* values were less than 5% ($P< 0.05$).

Results

The three possible rs2287499 genotypes were determined by sequencing the amplicons following PCR-SSCP [Figure-1]. Genotype and allele frequencies in patient and control groups did not display a deviation from Hardy-Weinberg equilibrium (P=0.98 &P= 0.07, respectively). In fact, the observed genotype distribution is a representative of the overall distribution in the Azeri population. In both groups, as expected, the (C) allele showed a higher frequency compared to the (G) allele (minor allele frequency = 0.19). However, difference in the variant allele's frequencies between cases and healthy individuals were found to be non-significant ($P>$ 0.05). In both groups, the dominant homozygotes (CC) were more widespread than heterozygotes (CG) and recessives homozygotes (GG), which is quite rare in the general population. Nonetheless, difference in genotype distributions between patients and controls was not statistically significant ($P>$ 0.05). According to the statistics, it seems that rs2287499

polymorphism has nothing to do with the risk of thyroid cancer among the Iranian-Azeri population (Table1). The possible prognostic importance of rs2287499 was also evaluated, and the sole significant association was observed in relation to the patient's gender (P=0.04). No further considerable association with remained clinicopathological parameters was evident (Table2).

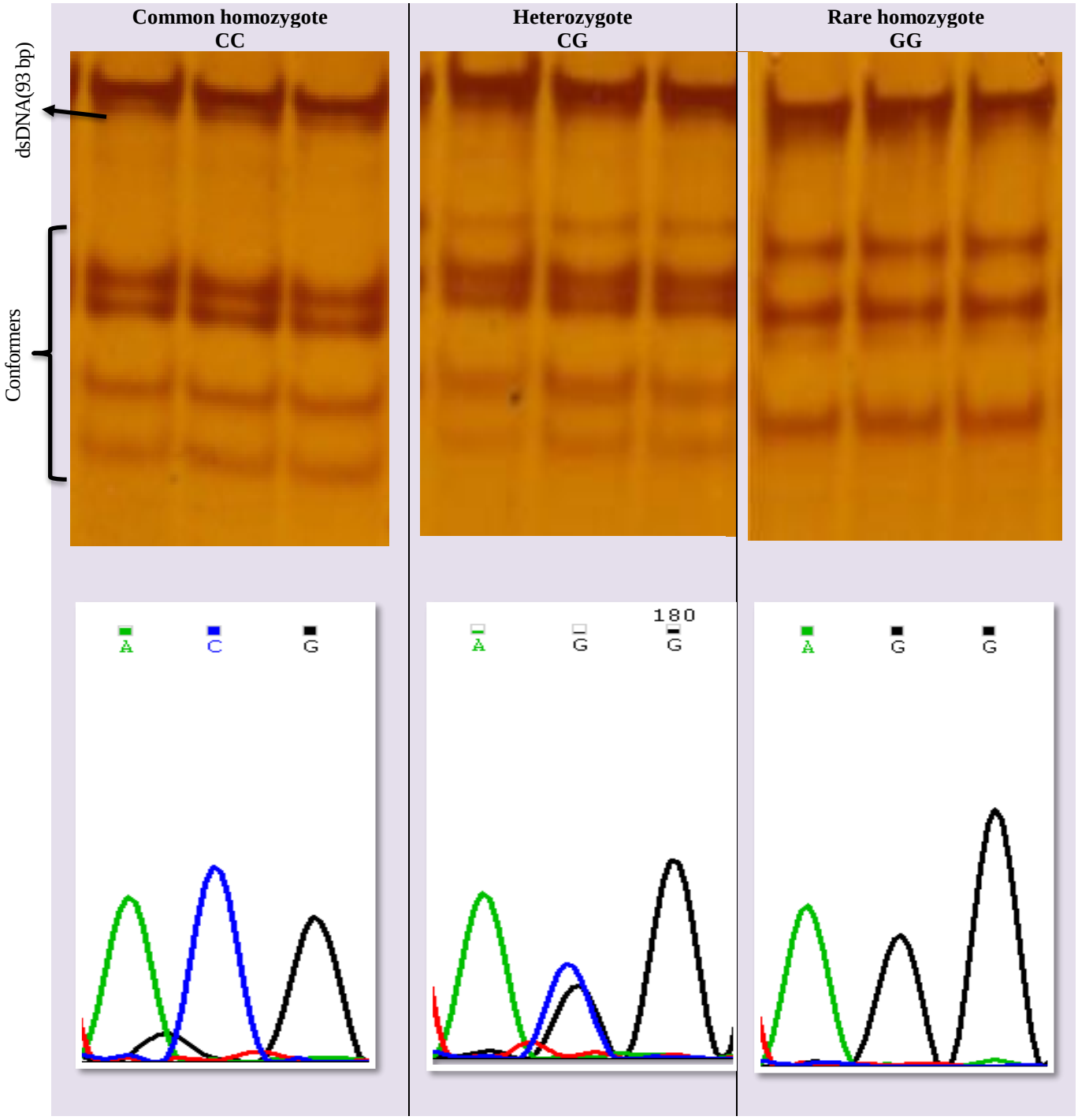

Figure 1. The allele-specific SSCP bands o006E polyacrylamide gel and the sequencing results of rs2287499 containing amplicons. Each banding pattern corresponds to a specific rs2287499 genotype. ACG codon encodes arginine wherase AGG encodes glycine.

Table 1. Genotype and allele frequencies.

rs2287499	Cases	Controls	OR (95% CI)	P value
Genotypes				
CC	79 (74.5%)	132 (67.3%)	1.41 (0.80- 2.49)	0.19
CG	25 (23.6%)	53 (27.1%)	0.83 (0.46- 1.49)	0.51
GG	2 (1.9%)	11 (5.6%)	0.32 (0.04- 1.58)	0.12
Alleles				
***C**	183 (86.3%)	317 (80.9%)	1.49 (0.91- 2.44)	0.09
G	29 (13.7%)	75 (19.1%)	0.67 (0.40- 1.09)	0.09

*C is the reference allele. Estimated relative risks with odds ratios (95% CI) and *P* values for association between rs2287499 and thyroid cancer risk.

Table 2. Clinicopathological characteristics of patients and estimated P values for SNP-disease associations by clinicopathological status.

Clinic-pathologic factors	CC	CG	GG	Total	P value
Age					
≤35	36 (34.0%)	14 (13.2%)	2 (1.9%)	52 (49.1%)	0.264
>35	43 (40.5%)	11 (10.4%)	0 (0.0%)	54 (50.9%)	
Gender					
Female	63 (59.4%)	18 (17.0%)	0 (0.0%)	81 (76.4%)	0.041
Male	16 (15.1%)	7 (6.6%)	2 (1.9%)	25 (23.6%)	
Tumor Type					
PTC	55 (51.9%)	14 (13.2%)	1 (0.9%)	70 (66.0%)	0.458
FTC	2 (1.9%)	3 (2.8%)	0 (0.0%)	5 (4.7%)	
MTC	2 (1.9%)	0 (0.0%)	0 (0.0%)	2 (1.9%)	
FTA	20 (18.9%)	8 (7.5%)	1 (0.9%)	29 (27.4%)	
Tumor Stage					
Early (I & II)	50 (58.7%)	16 (21.3%)	1 (1.3%)	67 (81.3%)	0.707
Late (III & IV)	7 (9.3%)	1 (1.3%)	0 (0.0%)	8 (10.7%)	
Tumor Size					
≤2cm	25 (38.5%)	5 (7.7%)	1 (1.5%)	31 (47.7%)	0.248
>2cm	24 (36.9%)	10 (15.4%)	0 (0.0%)	34 (52.3%)	
Involved Lobe					
Right	30 (36.1%)	11(13.3%)	1(1.2%)	42 (50.6%)	0.785
Left	24 (28.9%)	5(6.0%)	0 (0.0%)	29 (34.9%)	
Both	10 (12.1%)	2(2.4%)	0 (0.0%)	12 (14.5%)	
Lymph Node metastasis					
Positive	25 (34.7%)	7 (9.7%)	1 (1.4%)	33 (45.8%)	0.641
Negative	26 (40.6%)	5 (7.8%)	0 (0.0%)	39 (54.2%)	

(PTC: papillary thyroid carcinoma, FTC: follicular thyroid carcinoma, MTC: medullary thyroid carcinoma, FTA: follicular thyroid adenoma)

Discussion

The considerable prevalence of tumor-associated *TP53* mutations is a well-known phenomenon. Previous studies suggested that TP53 mutations may play an important role in thyroid cell malignant transformation and tumor progression. Nevertheless, inactivating mutations of p53 have been found to be less frequent in thyroid neoplasms than in other human malignancies. Mutant p53 was mainly detected inpoorly-differentiated and aggressive histotypes. Therefore, it is believed that this mutant protein is involved in prosression of

early stage tumors to more aggressive phenotypes and developing metastatic forms (34-37).

The anti-proliferative role of *TP53* could also be suppressed inwild-type p53 harboring tumors, implying other causes rather than mutations (38, 39). The efficiency of p53 tumor-suppressive activities may also be affected by genes surrounding this key anti-tumor, and this undeniably influences cancer susceptibility or treatment outcome (40). p53 mRNA degradation and failure in p53 induction upon DNA damage was evident after *WRAP53* knockdown, demonstrating the indispensable effects of *WRAP53* on p53 actions (14-17). Hence, dysfunction or lack of WRAP*53α* may contribute to loss of p53 function and eventually tumorigenesis of wt-p53-carrying cells.

UnlikeWRAP53*α*,whichexerts anti-oncogenic properties by regulating p53, WRAP53β gives rise to a potential onco-protein that exerts oncogenic activities when overexpressed. Enhanced WRAP53β levels have been detected in a broad range of human cancer cell lines (up to 20-fold higher) and primary tumors of ESCC (Esophageal Squamous Cell Carcinoma)(21), HNSCC (Head and Neck Squamous Cell Carcinoma)(18), parathyroid (22), brain (23), and colorectal (24), with a prognostic significance in HNSCC and rectal cancers. In particular, *WRAP53* was demonstrated to be a stronger prognostic factor than *TP53* in HNSSC (18). WRAP53β, as a key constituent of telomerase, is vital for telomere elongation in cells with active telomerase. Depletion of this protein disrupts telomerase-telomere association and leads to abrogation in telomere synthesis (19). Telomerase is principally activated in endocrine neoplasms, including thoseof the adrenal gland, the breasts, the prostate, the parathyroids, and the thyroid. Existence of improper active telomerase stabilizes telomere ends, which may ultimately promote cell immortalisation and tumorigenesis. Abberantly activated telomerase was predominantly detectable in malignant thyroid carcinomas, but not in adenomas and other benign lessions (19). These observations may further link WRAP53β to the risk of thyroid cancer.

However, variant alleles in the coding sequence of *WRAP53* could influence cancer susceptibility by impairing WRAP53α-mediated p53 regulation or converting amino acids in WRAP53β. Two common *WRAP53* polymorphisms located in *WRAP53-TP53* regulatory region, rs2287497 (an intronic change) and rs2287498 (Ex2- C >T- F150F), wereassociated with an increased risk of invasive ovarian cancer in Poland. This study showed that specific homozygosity of mentioned SNPs are significantly overrepresented in Polish ovarian cancer patients. The finding that these polymorphisms have stronger association with ovarian cancer risk than the frequent *TP53* rs1042522 (Ex4- C >G, P72R) SNP, was quite interesting (25). A strong association of rs2287498 with increased risk of serous ovarian cancer in non-hispanic white women was also established (26). In a mixed population from Poland and Norway, two linked SNPs in *WRAP53* "rs2287498 & rs2287499 (Ex1- C >G- R68G)" were significantly related to estrogen receptor-negative (but not ER-positive) breast cancer susceptibility. Conversely, anotable association with decreased breast cancer risk for *WRAP53* rs17885803 (IVS1-60, C >T) in Norwegian population was observed (27). The lack of rs2287499 association with breast cancer risk and prognosis in the Iranian-Azeri population was also reported in a recently published article (41). The exact influence of these SNPs on *WRAP53/TP53* functions has not been understood and needs to be elucidated.

There is also a possibility that the risk of thyroid tumor development may enhance through genetic variations in *WRAP53*. However, no evidence concerning the association of *WRAP53* SNPs with thyroid cancer risk and prognosis have been demonstrated so far. Inthis regard, we carried out a case-control association study to assess the relation between a prevalent sequence variation in *WRAP53*, rs2287499, and thyroid cancer risk and also prognosis among the Iranian-Azeri population residing in the northwest of IRAN. In both Azeri patients and control groups, the major dominant allele (C) was spotted more frequently than the alternative recessive allele (G). The widespread presence of dominant (CC) homozygotes and scarcity of recessive GG homozygotes werealso obvious in both groups. However, no evidence of a significant difference in the allele and genotpe frequencies

between cases and controls and thereforeno association of rs2287499 with a risk for developing thyroid tumor was observable in the studied population. Nevertheless, rs2287499 may still indirectly affect the risk for this disease. In case-only analysis, rs2287499 was significantly associated with gender, suggesting that there might be an indirect gender-based effect of rs2287499 on the risk of thyroid tumorigenesis. Indeed, there might have been an interaction between sex hormones and a specific rs2287499 genotype that contributed to the thyroid cancer susceptability in the Azeri population. As mentioned before, the rate of affection is higher in females, with a female-to-male occurance ratio of 2 in IRAN (42). Therefore, rs2287499 association with gender may partly account for gender differences in susceptibility to this cancer. However, we faild to detect an association betweenthis SNP with patient age as a potential thyroid cancer risk factor. Similarly, the association of rs2287499 genotypes with other prognostic indicators, including tumor type & size, stage of the disease, involved lobe, and lymph node metastases were not established to be significant. This means that rs2287499 cannot be evaluated to predict the disease progression according to the status of the mentioned parameters.

To provide a deeper insight into the role of rs2287499 in the pathogenesis of human cancers, its association with other malignancies in a larger spectrum of specimens needs to be surveyed. The potential functional significance of rs2287499 and also its regulatory effect on *TP53* expression would also be of interest and clearly warrant further investigations.

Although the *WRAP53* rs2287499 variation may affect the risk of thyroid cancer in a gender-based manner, it does not directly lead to thyroid cancer susceptibility in the general Azeri population of IRAN. The studied SNP is not a molecular biomarker for thyroid cancer risk and cannot serve as a prognostic biomarker to evaluate the disease progression regarding clinical and pathologic features. This work is based on the Master's thesis of the first author, and due to time restrictions, a limited number of specimens havebeen examined. However, it isworth carrying out further studies in various populations of IRAN and with a higher number of samples.

Declarations of interest

No conflicts of interest exist for this paper.

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