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The BRAF, NRAS Mutations and Clinic-pathological features of Thyroid Tumors in Mongolia

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Abstract

Objective: In this study, we aimed to study BRAFV600E and NRAS mutations among thyroid tumor patients in the Mongolian population.

Methods: Immunohistochemical staining was performed using CD56 antibodies on 59 formalin-fixed paraffin-embedded (FFPE) tissue sections. DNA extractions from FFPE and fresh thyroid tumor tissues were extracted using a genomic DNA kit. An ABI 3730xl genetic analyzer was used for DNA sequencing.

Results: The thyroid tumors of 59 patients were studied. These patients were predominantly female (53, 89.8%), and the mean age was 45.61±14.2 years (range 13–72 years). Of these cases, 46 (78%), 3 (5.1%), and 8 (13.61%) were diagnosed as PTC, FTC, and follicular adenoma, respectively, by histology. Immunohistochemistry analysis found that CD56 was expressed in 7 (87.5%) benign tumors and 14 (27.5%) malignant tumors, and the difference was significant (p=0.002). In cases of PTC, the BRAFV600E mutation was positive in 9 (19.6%) cases, while the NRAS mutation was positive in 2 (3.38%) cases. However, the manifestations of these mutations are not significantly associated with the clinic pathological features of PTC.

Conclusion: BRAFV600E and NRAS mutations occurred at a relatively low rate and were not correlated with increased PTC aggressiveness in our study.

Keywords: BRAFV600E; NRAS; Mutation; Thyroid papillary carcinoma; Follicular adenoma; Clinic pathological features; Thyroid tumors

Introduction

Thyroid cancer is the most common endocrine malignancy and presents in 5–30% of total thyroid nodule. It can be classified into papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), medullary thyroid carcinoma (MTC), and poorly differentiated, undifferentiated or anaplastic carcinoma. The great majority of thyroid cancers are PTC, representing approximately 65–85% of total thyroid cancers. Recently, the incidence of thyroid tumors has gradually increased in Mongolia, doubling in the last 10 years [1-4].

Several genetic mutations have been detected in thyroid tumors, among which BRAF and RAS mutations are well known. BRAF mutations occur in approximately 8% of all types of cancers, with a high mutation frequency in malignant melanoma (50–70%), classic PTC (40–70%), colorectal cancer (CRC, 5–15%), ovarian cancer and hairy cell leukemia (5–100%). The c.1799T>A mutation results in a valine-to-glutamate substitution (p.V600E) at amino acid position 600. This prevalent mutation accounts for over 80% of all BRAF mutations and almost every mutation in thyroid tumors [5-9].

The RAS mutation is found in 10 to 20% of PTCs, but this mutation is slightly more common in follicular neoplasms, including follicular carcinoma (40–50%) and follicular adenoma (20–40%). Identifying these mutations is important for choosing a suitable type of treatment, predicting disease prognosis, and providing detailed diagnostic information. However, no studies have been performed to assess the frequency of BRAF and RAS mutations in thyroid tumors in the Mongolian population [10-13].

CD56 is a neural cell adhesion molecule (NCAM) that mediates homotypic and heterotypic cell-cell adhesion through homophilic binding mechanisms and is widely used in the differential diagnosis of follicular neoplasms. In addition, CD56 is considered a prognostic marker in various cancers, including pancreatic cancer, colon carcinoma and astrocytoma. In cases of thyroid cancers, the expression of CD56 is diminished and correlates with metastatic potential and poor prognosis in some malignant tumors [14].

Therefore, we aimed to study BRAFV600E and NRAS mutations among patients with thyroid tumors and their correlations with histopathological features and CD56 expression related to the prognostic profile in the Mongolian population [15-17].

Materials and Methods

Sample source

We collected 9 fresh thyroid tissue samples and 50 formalin-fixed paraffin-embedded (FFPE) tissue samples from patients who underwent radical surgery at the National Cancer Center (NCC) of Mongolia from 2017 to 2019. All pathologic slides, along with the pathologic reports and clinical records, were reviewed by two expert pathologists [18].

Immunohistochemical staining and evaluation

Staining methods

Immunohistochemical staining was performed using primary

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antibodies against CD56 (mouse, 123C3.D5, dilution 1:500, Sigma–Aldrich, USA) on FFPE sections. Membranous staining of follicular cells with CD56 was considered positive immunohistochemical staining. The modified Allred scoring system was used to evaluate positivity, with staining intensity and distribution scored separately [19]. The staining intensity was scored as 0 (negative), 1 (weak), 2 (intermediate), or 3 (strong), and the distribution of positively stained cells was semi quantitatively graded as 0 (negative) or 1 (67% positively stained cells). The total staining score was calculated as the sum of these two parameters. Total staining scores from 0 to 2 points were considered negative, while scores from 3 to 8 points were considered positive. To overcome the limitation of this quantification method, we compared the mean staining score as a continuous variable [20-22].

DNA extraction

DNA was extracted from 3–5 sections of FFPE tissues (3–5 microns/section). The sections were deparaffinized by xylene and ethanol treatment and digested with proteinase K at 56 °C overnight. Genomic DNA was extracted using a genomic DNA I kit (TIANamp FFPE DNA kit, Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. DNA extracted from fresh thyroid tumor tissue was extracted using a genomic DNA I kit (Choros Onosh, Ulaanbaatar, Mongolia) as recommended [23].

Polymerase chain reaction (PCR) and sequencing

PCR was performed with primers designed to amplify BRAF exon 15 per Frasca et al. (2008): forward TCATAATGCTTGCTCTGATAGGA and reverse GGCCAAAAATTTAATCAGTGGA.

NRAS exon 3 was evaluated by PCR using primers previously described by Marina et al. (2002): forward 5`- CCT GTT TGT TGG ACA TAC TG -3` and reverse 5`- CCT GTA GAG GTT AAT ATC CG-3`[15]. PCR was performed using standard conditions (95 °C for 5 min, 94 °C for 30 sec, 58 °C for 30 sec, 72 °C for 30 sec, for 40 cycles; 70 °C for 10 min). PCR fragments were analyzed by 2% agarose gel electrophoresis [24].

A DNA cycle sequencing reaction with a total volume of 10 µl was performed on a thermal cycler using 5 µl of Zacdye 2X premix, 1 µl of PCR product (50 ng/ul), 1 µl of primer of 1 µM and 2 µl of water. The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 30 repetitive cycles of 95 °C/20 sec, 52 °C/30 sec and 60 °C/2 min. Reaction products were purified by sodium acetate and ethanol precipitation, dissolved in formamide DNA solvent (Zanaspex) and loaded into an ABI 3730xl genetic analyzer. Sequencing data were analyzed by Codon Code Alginer ver.9.0.1 software [25-27].

Statistical analysis

Analyses were performed using the IBM SPSS V25.0 program (SPSS Inc., Chicago, USA). Categorical variables were compared using chi-square or Fisher's exact tests. The Shapiro–Wilks test was used to assess the normality of the distribution of continuous variables. The Mann–Whitney U test was used to evaluate differences in continuous variables between two independent groups. Binary logistic regression analysis was used to assess the independent association of BRAF and NRAS mutations. In these tests, p values were 2-sided, and statistical significance was defined as $p < 0.05$ [28].

Ethics approval

The institutional review board of the Mongolian National University of Medical Sciences (MNUMS) approved this study (2017.06.09, №17/03-08).

Results

Clinic pathological features

The thyroid tumors of 59 patients were studied. The patients were predominantly female (53, 89.8%), and the mean age was 45.61±14.2 years (range 13–72 years). The histopathological diagnosis of thyroid tumors identified PTC in 46 cases (78%), FTC in 4 (6.8%), follicular adenoma (FA) in 8 (13.61%), and MTC in 1 (1.7%). The median tumor size was 1.5 cm (range 0.25–10.00). Among the 46 PTC cases, 38 (82.6%) were classic, 6 (13%) were follicular variants, 1 (2.2%) was a tall-cell variant, and 1 (2.2%) was an oncocytic variant [29].

Of the 31 (60.8%) patients diagnosed with PTCs at pathologic tumor stages I-II, 20 (64.5%) showed lymphatic vessel invasion, 17 (54.8%) metastasized to regional lymph nodes, and 20 (64.5%) were invasive to the tumor capsule. Of the 15 (32.6%) patients diagnosed with PTCs at pathologic tumor stages III-IV, 13 (86.7%) showed lymphatic vessel invasion, 12 (80%) metastasized to regional lymph nodes, and 11 (73.3%) had extra thyroidal invasion. Generally, the pathologic T stage was associated with extra thyroidal invasion (p=0.02) [30].

CD56 expression in thyroid tumors

CD56 expression was detected by immunohistochemistry in 7 (87.5%) and 14 (27.5%) benign and malignant tumors, respectively (Figure 1). This difference in CD56 expression was statistically significant between tumors of benign and malignant origins (p=0.002). For PTC alone, CD56 was expressed in 12 (26%) and not expressed in 34 (73.9%), including 27 (58.7%) classic and 7 (15.2%) other variants. However, no significant difference in the expression of CD56 was observed between classic and other variants of PTC (p=0.66) [31].

Furthermore, there were no significant differences in other clinic pathological features, including tumor size, capsular invasion, tumor location, extra thyroid invasion, lymphatic invasion and regional lymph node metastasis, between these 2 groups (CD56 negative vs. positive) (p>0.05) [32].

BRAF V600E and RAS mutations in thyroid tumors

In our study, a total of 10 (16.9%) cases with thyroid tumors were determined to have the BRAFV600E mutation, of which 9 (90%)

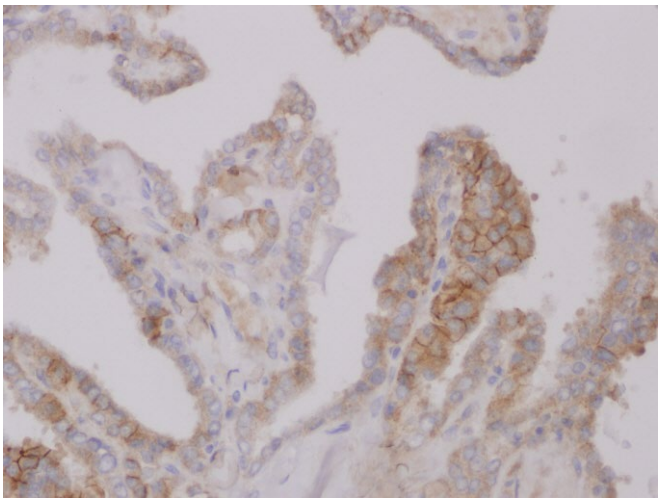

Figure 1: The CD56 expression in papillary thyroid carcinoma. The membranous staining of CD56 is considered as positive in the tumor cells.

were diagnosed with PTC, and 1 (10%) was diagnosed with follicular adenoma. All of these mutations were heterozygous (Figure 2). Of the 9 cases with the BRAFV600E mutation, 8 were women, and 5 were older than 45. However, the mutation was not associated with patient age or sex (p>005) [33].

All CD56-positive cases of PTC with the BRAFV600E mutation were classical variants. Of these, 8 (25.8) were more than 1 cm in size, 5 (16.1%) were stage TI-II, 8 (24.2%) had vascular invasion, 8 (24.2%) had lymphatic vessel invasion, and 6 (17.6%) had extra thyroidal invasion. However, there was no statistically significant difference in the presence of the BRAFV600E mutation between the CD56-positive and CD56-negative groups (Table 1) [30].

Heterozygous BRAFV600E mutations were detected in 8 CD56-negative cases and 1 CD56-positive case. No significant differences in the BRAF mutation and CD56 expression were shown to be associated with prognosis (Table 1) [31].

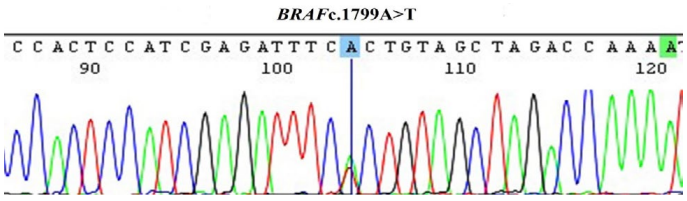

Figure 2: Detection of BRAFV600E mutation in papillary thyroid carcinoma.

The risk factors for the BRAFV600E mutation were explored. (Table 2) shows the ORs for positive expression of CD56 (OR=0.295, 95%CI=0.033–2.654, p=0.276, (Figure 1), pathologic tumor stage III-IV (OR=1.891, 95%CI=0.425–8.406, p=0.403), tumor size greater than 1 cm (OR=4.87, 95%CI=0.549–43.183, p=0.155), presence of lymphatic vascular invasion (OR=3.84, 95%CI=0.43–34.306, p=0.229), presence of tumor capsular invasion (OR=3.84, 95%CI=0.43–34.306, p=0.229), presence of extra thyroidal extension (OR=1.75, 95%CI=0.356–8.609, p=0.491), and regional lymph node invasion (OR=1.217, 95%CI=0.262–5.661, p=0.802) [32].

Of the 59 cases examined in the study, the NRAS mutation was positive in only 2 (3.4%) cases of PTC (Figure 3). Both cases of NRAS mutations were classic variants of PTC that did not extend to the extra thyroidal region, and one of these cases was CD56-positive and metastatic to the lymph nodes. However, no significant differences were found between patients with or without NRAS mutations [33].

Discussion

According to the World Health Organization (WHO) annual report, 532,000 new cases of thyroid cancer were diagnosed in 2020, ranking ninth in the total number of cancer cases. In 2020, the number

Table 1: BRAFV600E mutation and clinic pathological features in papillary thyroid carcinoma.

		Total	BRAF				P value
			Negative		Positive		
		N	n (%)	95% CI	n (%)	95% CI	
Age group							0.718
	45≤	22	17 (77.3)	57.1 - 90.8	5 (22.7)	9.2 - 42.9	
	45>	24	20 (83.3)	65.1 - 94.1	4 (16.7)	5.9 - 34.9	
Gender							0.848
	Female	40	32 (80)	65.8 - 90.1	8 (20)	9.9 - 34.2	
	Male	6	5 (83.3)	44.2 - 98.1	1 (16.7)	1.9 - 55.8	
CD 56							0.409
	Negative	34	26 (76.5)	60.5 - 88.2	8 (23.5)	11.8 - 39.5	
	Positive	12	11 (91.7)	67.2 - 99.1	1 (8.3)	0.9 - 32.8	
Tumor size							0.235
	1≤	15	14 (93.3)	72.8 - 99.3	1 (6.7)	0.7 - 27.2	
	1>	31	23 (74.2)	57.1 - 87	8 (25.8)	13- 42.9	
Tumor Stage							0.445
	I-II	31	26 (83.9)	68.2-93.6%	5 (16.1)	6.4-31.8%	
	III-IV	15	11 (73.3)	48.3-90.3%	4 (26.7	9.7-51.7%	
Vascular invasion							0.41
	No	13	12 (92.3)	69.3-99.2%	1 (7.7)	0.8-30.7%	
	Yes	33	25 (75.8)	59.4-87.8%	8 (24.2)	12.2-40.6%	
Capsular invasion							0.41
	No	13	12 (92.3)	69.3-99.2%	1 (7.7)	0.8-30.7%	
	Yes	33	25 (75.8)	59.4-87.8%	8 (24.2)	12.2-40.6%	
Extra-thyroid invasion							0.666
	No	34	28 (82.4)	67.2 - 92.3	6 (17.6)	7.7 - 32.8	
	Yes	11	8 (72.7)	43.5 - 91.7	3 (27.3)	8.3 - 56.5	
Tumor location							0.637
	Unilateral	21	16 (76.2)	55.4 - 90.3	5 (23.8)	9.7 - 44.6	
	Bilateral	9	8 (88.9)	58.6 - 98.8	1 (11.1)	1.2 - 41.4	
Lymph nodal metastasis							0.802
	No	17	14 (82.4)	60-94.8	3 (17.6)	5.2-40%	
	Yes	29	23 (79.3)	62.2-90.9%	6 (20.7)	9.1-37.8%	
Total		46	37 (80.4)	67.3 - 89.9	9 (19.6)	10.1 - 32.7	

Table 2: The risk factor evaluation of BRAFV600E mutation.

Variables	BRAF(+)/ normal		P value
	OR	95% CI	
CD 56 (+)	0.295	0.033 - 2.654	0.276
Tumor stage III-IV	1.891	0.425-8.406	0.403
Tumor size (>1cm)	4.87	0.549 - 43.183	0.155
Vascular invasion (+)	3.84	0.43-34.306	0.229
Capsular invasion (+)	3.84	0.43-34.306	0.229
Extra-thyroid invasion (+)	1.75	0.356 - 8.609	0.491
Lymph node metastasis (+)	1.217	0.262-5.661	0.802

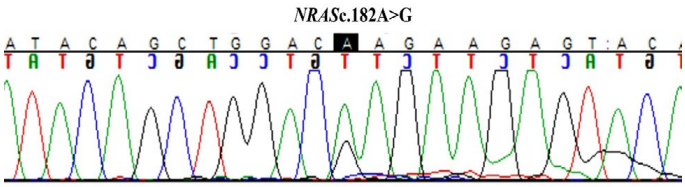

Figure 3: Detection of NRAS mutation in papillary thyroid carcinoma.

of deaths from thyroid cancer was 43,646, making it the 24th leading cause of cancer-related deaths. By region, the prevalence of thyroid cancer is 59.7% in Asia, 20% in North and South America, and 15% in Europe, with the highest rates in Korea, Canada, and Italy. According to the statistics of the Mongolian National Cancer Center, 32 new cases of thyroid cancer were registered in 2010, but 73 new cases were diagnosed in 2020 [34].

In our study, we included 59 patients diagnosed with thyroid tumors who underwent surgery, and most of these patients (78%) had PTC. The classic variants of PTC were the most prevalent variant and were usually diagnosed in pathologic stages I-II. Although diagnosed early, 33 (71.7%) of all PTCs showed foci of lymphatic vessels and tumor capsule invasion. Among them, 11 (23.9%) cases infiltrated the extra thyroidal soft tissue, and 29 (63%) cases metastasized to the regional lymph nodes [35].

CD56 expression was detected by immunohistochemistry in 87.5% of benign tumors and 27.4% of malignant tumors. This difference in CD56 expression was statistically significant (p=0.002). For PTC alone, CD56 was not expressed in 73.9% (n=34) of cases, including 58.7 % (n=27) of classic cases and 15.2% (n=7) of other variants. These results are similar to those of other previous studies, such as Dunderovic et al. (2015). CD56 expression is correlated with metastatic potential and poor prognosis in some malignant tumors. However, CD56 was considered important in diagnosing follicular neoplasms to distinguish between benign and malignant potential, although we did not significance of these clinic pathological findings related to CD56 expression [36].

In this study, the BRAF V600E mutation was detected in 19.6% of thyroid papillary carcinomas, which is relatively less than reported in previous studies. For example, the BRAFV600E mutation was detected in 31.6% and 86.7% of thyroid papillary carcinomas in Stanojevic et al. (2011) and Huang et al. (2019), respectively. Thus, BRAF mutations may be commonly detected in countries such as Korea, China, Australia and Argentina, with relatively high thyroid cancer incidence rates. Our study has some limitations that may have influenced our results [37]. Because Mongolia has a small population of 3 million, the incidence of thyroid cancer is lower than in countries with large populations, so it was possible to include a small number of specimens in our study. The National Cancer Center is the only major cancer surgery hospital in Mongolia, and we did not include cases of inadequate patient information and poor quality of stored paraffin blocks. In addition, most of the materials used in our study contained FFPE tissue, which may have influenced the sequencing results. Furthermore, BRAF mutations were detected in thyroid cancer at a lower rate than in other countries, possibly due to the low radiation exposure in Mongolia. However, there is still a lack of research on the risk and causes of thyroid cancer in Mongolia [38].

In this study, no significant differences were found in sex, age, tumor size and multimodality between patients with or without BRAFV600E mutations, similar to the findings of multiple previous studies. In the abovementioned studies, the BRAFV600E mutation was typically identified in classical and tall cell variants. However, it was rarely identified in the follicular variant. Additionally, in our study, all cases with the BRAFV600E mutation were related to classical variants. On the other hand, there were differences in histological type, lymph node metastasis, tumor stage, and extra thyroidal extension between these 2 groups in other studies. However, these differences were not observed in our study [39].

There was a correlation between the BRAFV600E mutation and increased PTC aggressiveness in studies carried out in the USA6), Italy, Turkey. However, there was no correlation found between the BRAFV600E mutation and increased PTC aggressiveness in studies carried out in Japan, Korea Taiwan, France, Russia and some studies in Italy. In addition, there was no correlation found in our study.

In our study, NRAS mutations were detected in 3.38% of PTCs, which is much less than other studies reported. Additionally, there was no correlation between the clinic pathological features and NRAS mutation (exon 3) in our study or other studies [40].

Conclusion

In conclusion, BRAFV600E and NRAS mutations were detected in 19.6% and 3.38% of PTC cases, respectively, in our study. We found no correlation between BRAFV600E and NRAS mutations and increased PTC aggressiveness.

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