K-RAS and *N-RAS* mutations in testicular germ cell tumors

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ABSTRACT

Testicular cancer is a relatively rare tumor type, accounting for approximately 1% of all cancers in men. However, among men aged between 15 and 40 years, testicular cancer is the most commonly diagnosed malignancy. Testicular germ cell tumors (TGCTs) are classified as seminoma and non-seminoma. The *RAS* oncogene controls several cellular functions, including cell proliferation, apoptosis, migration, and differentiation. Thus, RAS signaling is important for normal germ cell development. Mutations of the Kirsten RAS (*K-RAS*) gene are present in over 20% of all cancers. *RAS* gene mutations have also been reported in TGCTs. We investigated *K-RAS* and *N-RAS* mutations in seminoma and non-seminoma TGCT patients. A total of 24 (55%) pure seminoma cases and 19 (45%) non-seminoma cases were included in the study. *K-RAS* and *N-RAS* analyses were performed in our molecular pathology laboratory, using *K-RAS* and *N-RAS* Pyro Kit 24 V1 (Qiagen). In total, a *RAS* mutation was present in 12 patients (27%): 7 seminoma (29%) and 5 non-seminoma cases (26%) [p = 0.55]. A *K-RAS* mutation was present in 4 pure seminoma tumors (16%) and 3 non-seminoma tumors (15%) [p = 0.63], and an *N-RAS* mutation was observed in 4 seminoma tumors (16%) and 3 non-seminoma tumors (15%) [p = 0.63]. Both, *K-RAS* and *N-RAS* mutations were present in two patients: One with seminoma tumor and the other with non-seminoma tumor. To date, no approved targeted therapy is available for the treatment of TGCTs. The analysis of *K-RAS* and *N-RAS* mutations in these tumors may provide more treatment options, especially in platinum-resistant tumors.

KEY WORDS: Testicular germ cell tumors; seminoma; non-seminoma; *K-RAS* mutation; *N-RAS* mutation; TGCT
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INTRODUCTION

Testicular cancer is a relatively rare tumor type, accounting for approximately 1% of all cancers in men. However, among men aged between 15 and 40 years, testicular cancer is the most commonly diagnosed malignancy [1]. Furthermore, roughly 95% of all malignant tumors of the testis are germ cell tumors. Although these tumors can also arise in extragonadal primary sites, they are still managed in the same way as testicular germ cell tumors (TGCTs) [2]. TGCTs are classified into two groups: Seminoma and non-seminoma tumors. Non-seminoma tumors include several subtypes of cancers, including embryonal cell carcinoma, choriocarcinoma, yolk sac tumor, and teratoma. Serum tumor markers such as alpha-fetoprotein (AFP), lactate dehydrogenase (LDH), and beta-human chorionic gonadotropin (beta-hCG) are critical in diagnosing TGCTs, determining prognosis, and assessing treatment outcomes [3].

It has been shown that TGCTs are highly sensitive to radiation and chemotherapy, and consequently, the cure rate is high in these cases. However, approximately 5% of patients develop resistance to the treatment [4].

The *RAS* oncogene controls several cellular functions, including cell proliferation, apoptosis, migration, and differentiation. Thus, RAS signaling is important for normal germ cell development [5]. Kirsten RAS (*K-RAS*) mutations are present in over 20% of all cancers [6]. Although *RAS* gene mutations have also been reported in TGCTs [7], the role of RAS pathway alterations in the treatment response is still not clear. In addition, seminomas and non-seminomas may also occur as mixed germ cell tumors, but in clinical management, they are

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categorized as non-seminomas. In this study, we investigated *K-RAS* and *N-RAS* mutations in seminoma and non-seminoma TGCT patients, aiming to determine the relationship between these mutations and pathologic factors, prognostic factors, and response to therapy.

MATERIALS AND METHODS

Patients and samples

We retrospectively reviewed the records of patients diagnosed with TGCT between 2007 and 2015 at Trakya University Medical Oncology Department. Paraffin-embedded tissue blocks from these patients were collected from the archive of our pathology department. Twenty-four pure seminoma cases and 19 non-seminoma cases were included in the study. All tumoral slides were reevaluated and the pathologic diagnoses were confirmed by a pathologist. Cancer staging was performed according to the TNM Classification of Malignant Tumors (TNM staging) in the Seventh Edition American Joint Committee on Cancer (AJCC) Cancer Staging Manual. The study was approved by the Institutional Ethics Board. Informed consent was obtained from all individual participants or their legal representatives.

Mutation analysis

K-RAS and *N-RAS* analyses were performed in our molecular pathology laboratory, and the following mutations were analyzed: CAA>CTA Q61L, CAA>CAT Q61H, CAA>CGA Q61R, GGT>GTT G12V, GGC>GAC G13D, GGT>GAT G12D, GGT>TGT G12C, GGT>AGT G12S, GGT>GCT G12A, GGT>CGT G12R, and GGT>c.34_35GG>TT G12F. DNA isolation was performed from the primary tumor tissue samples using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). The Pyro Kit 24 V1 (Qiagen, Hilden, Germany) was used for *K-RAS* and *N-RAS* analyses, and *K-RAS* and *N-RAS* point mutations were analyzed with the PyroMark Q24 Software System (Qiagen, Hilden, Germany).

Statistical analysis

We used IBM Statistical Package for Social Sciences (SPSS, Chicago, IL, USA) version 18.0 to perform statistical analyses in this study. Overall, patient survival was calculated from the date of diagnosis to date of death from the disease or to the patient's last follow-up. The relationship between non-parametric variables was studied using Chi-square test, and parametric variables were compared using an independent samples *t*-test. The value of $p < 0.05$ was considered statistically significant. Survival estimates were calculated using the Kaplan-Meier method.

RESULTS

Clinical-pathological features and TNM staging of the cases are shown in Tables 1 and 2.

Overall, 24 patients (55%) were diagnosed with pure seminoma tumors and 19 patients (45%) were diagnosed with non-seminoma tumors. The median age of participants was 30 years (min: 19 - max: 63). In total, a *RAS* mutation was present in 12 tumors (27%): 7 pure seminoma cases (29%) and 5 non-seminoma cases (26%) ($p = 0.55$). A *K-RAS* mutation was present in 4 pure seminoma tumors (16%) and 3 non-seminoma tumors (15%) ($p = 0.63$); an *N-RAS* mutation was also present in 4 pure seminoma tumors (16%) and 3 non-seminoma tumors (15%) ($p = 0.63$). While all of the *N-RAS* mutations were 181 C>A (Q61K) in the codon 61 of the exon 3, *K-RAS* mutations were detected in different codons. The most common *K-RAS* mutation was 436G>A (A146T) in the codon 146 of the exon 4 (in 3 cases). In addition, two patients had GGT>AGT (G12S) mutation in the codon 12 of the exon 2, one patient had GGC>GAC (G13D) mutation in the codon 13 of the exon 2, and another had CAA>CTA (Q61L) mutation in the codon 61 of the exon 3.

Furthermore, a total of 19 patients (44%) had lymphovascular invasion (LVI) at the time of diagnosis. There was no significant difference between RAS mutant and wild-type patients in the rate of LVI. Moreover, involvement of tunica albuginea was observed in 12 patients (28%) and this was not associated with the presence of *RAS* mutations. Other pathologic features of the testes (i.e., tunica vaginalis, spermatic cord, and scrotal invasion) were not statistically different between the *RAS* wild-type and mutant patients ($p = 0.61$).

It should be noted that two patients involved in the study died before the time of this analysis. Both patients had *RAS* wild-type tumors. One tumor was seminoma and the other was non-seminoma. The patient who had seminoma tumor did not receive adjuvant chemotherapy but did receive adjuvant radiotherapy. The overall survival was 14 months for this patient. The patient with non-seminoma died 31 months after the diagnosis. Aside from these two cases, the progression was observed in four patients. Three of these patients had seminoma tumor and the fourth had non-seminoma tumor. Only in one of these patients CAA>CTA (Q61L) mutation was observed in the *K-RAS* codon 61. This patient received a first-line combination chemotherapy containing bleomycin, etoposide, and cisplatin (BEP). Following the progression, he received cisplatin, etoposide, and ifosfamide as the second-line chemotherapy. The other patients received BEP therapy at the time of progression. After the second-line chemotherapy, recurrence or metastasis did not occur in these patients. They were still alive at the time of writing this manuscript. In our study, there was a limited number of events for the overall and progression-free survival analyses.

TABLE 1. Comparison of clinical and tumor pathological characteristics according to *RAS* mutations

Clinical and pathological characteristics of patients with TGCT	Extended-*RAS* mutant n (%)	Extended-*RAS* wild-type n (%)	*p*	*K-RAS* mutant n (%)	*K-RAS* wild-type n (%)	*p*	*N-RAS* mutant n (%)	*N-RAS* wild-type n (%)	*p*
Tumor type									
Seminoma+non-seminoma	12 (27.9)	31 (72.1)		7 (16.3)	36 (83.7)		7 (16.3)	36 (83.7)	
Seminoma	7 (16.3)	17 (39.5)	1.0	4 (16.7)	20 (83.3)	0.635	4 (16.7)	20 (83.3)	0.635
Non-seminoma	5 (11.6)	14 (32.6)		3 (15.8)	16 (84.2)		3 (15.8)	16 (84.2)	
Side									
Right	6 (50.0)	12 (38.7)	0.687	3 (7.0)	15 (34.9)	0.905	3 (7.0)	15 (34.9)	0.905
Left	6 (50.0)	18 (58.1)		4 (9.3)	20 (46.5)		4 (9.3)	20 (46.5)	
Bilateral	0 (0)	1 (3.2)		0 (0)	1 (2.3)		0 (0)	1 (2.3)	
Lymphovascular invasion									
Present	5 (11.6)	14 (32.6)	1.0	3 (7.0)	16 (37.2)	0.635	3 (7.0)	16 (37.2)	0.635
Absent	7 (16.3)	17 (39.5)		4 (9.3)	20 (46.5)		4 (9.3)	20 (46.5)	
Tunica albuginea invasion									
Present	3 (7.0)	9 (20.9)	1.0	3 (7.0)	9 (20.9)	0.296	0 (0)	12 (27.9)	0.082
Absent	9 (20.9)	22 (51.2)		4 (9.3)	27 (62.8)		7 (16.3)	24 (55.8)	
Tunica vaginalis invasion									
Present	1 (2.3)	2 (4.7)	1.0	1 (2.3)	2 (4.7)	0.421	0 (0)	3 (7.0)	0.491
Absent	11 (25.6)	29 (67.4)		6 (14.0)	34 (79.1)		7 (16.3)	33 (76.7)	
Spermatic cord invasion									
Present	0 (0)	1 (2.3)	1.0	0 (0)	1 (2.3)	0.837	0 (0)	1 (2.3)	0.837
Absent	12 (27.9)	30 (69.8)		7 (16.3)	35 (81.4)		7 (16.3)	35 (81.4)	
Scrotal invasion									
Present	0 (0)	1 (2.3)	0.721	0 (0)	1 (2.3)	0.837	0 (0)	1 (2.3)	0.837
Absent	12 (27.9)	30 (69.8)		7 (16.3)	35 (81.4)		7 (16.3)	35 (81.4)	
N stage									
Nod positive	3 (7.0)	9 (20.9)	0.555	3 (7)	9 (20.9)	0.378	1 (2.3)	11 (25.6)	0.652
Nod negative	9 (20.9)	22 (51.2)		4 (9.3)	27 (62.8)		6 (14.0)	25 (58.1)	
Metastasis									
Present	1 (2.3)	5 (11.6)	0.659	1 (2.3)	5 (11.6)	0.681	0 (0)	6 (14.0)	0.319
Absent	11 (25.6)	26 (60.5)		6 (14.0)	31 (82.1)		7 (16.3)	30 (69.8)	
Risk classification									
Good	12 (100)	2 (6.5)	0.515	7 (16.3)	34 (79.1)	0.698	7 (16.3)	34 (79.1)	0.698
Medium and poor	0 (0)	29 (93.5)		0 (0)	2 (4.7)		0 (0)	2 (4.7)	

TGCTs: Testicular germ cell tumors

TABLE 2. TNM classification of patients with TGCT

TNM classification	Stage	n (%)
T1 N0 M0 S0	1A	18 (41.9)
T2 N0 M0 S0	1B	9 (20.9)
T1 N0 M0 S1	1S	2 (4.7)
T1 N1 M0 S0	2A	1 (2.3)
T2 N1 M0 S0	2A	1 (2.3)
T4 N1 M0 S0	2A	1 (2.3)
T2 N2 M0 S0	2B	3 (7.0)
T1 N3 M0 S0	2C	1 (2.3)
T2 N M0 $M1_A$ S0	3A	1 (2.3)
T1 N1 $M1_A$ S0	3A	1 (2.3)
T2 N2 $M1_A$ S0	3A	1 (2.3)
T2 N3 $M1_A$ S0	3A	2 (4.7)
T2 N3 $M1_A$ S1	3A	1 (2.3)
T1 N3 M0 S2	3B	1 (2.3)

TGCTs: Testicular germ cell tumors TNM: TNM classification of malignant tumors

DISCUSSION

A distinguishing characteristic of TGCTs is a mutation on the short arm of chromosome 12 (12p), identified in almost all invasive TGCTs, as well as in intratubular embryonal carcinoma and intratubular seminoma [8,9]. K-RAS and N-RAS are components of the Ras-Raf-MEK-ERK pathway. K-RAS and N-RAS mutations in the codons 12, 13, and 61 cause inappropriate activation of the pathway independent of epidermal growth factor-epidermal growth factor receptor (EGF-EGFR) binding. The constitutively active Ras-Raf-MEK-ERK pathway initiates carcinogenesis by promoting cellular proliferation, gene expression, differentiation, mitosis, and cell survival. Patients with lung and colorectal cancers who have these mutations showed unfavorable response to anti-EGFR-targeted or receptor tyrosine kinase inhibitor (TKI) therapy [10,11].

In the COSMIC study, *RAS* mutations (*K-RAS* [13/214] and *N-RAS* [8/145]) were observed in approximately 6% of seminomas; both types of mutations were absent in non-seminomas in this study (*K-RAS* [0/138] and *N-RAS* [0/71]) [6]. In another study, Sommerer et al. [12] identified *K-RAS* mutations in 7% of seminomas (2/30) and 9% of non-seminoma TGCTs (3/32) [12]. Furthermore, Ganguly et al. [13] found

that 59% of seminomas (13/22) and 78% of non-seminomas (7/9) had *N-RAS* gene mutations [13]. In these studies, none of the patients had both *K-RAS* and *N-RAS* mutations. In previous studies, the incidence of *K-RAS* and *N-RAS* mutations in TGCTs was 6-7%; however, it was 27.9% in our study. In the present study, a *K-RAS* mutation was present in 4 pure seminoma tumors (16%) and 3 non-seminomas (15%) and an *N-RAS* mutation was present in 4 seminomas (16%) and in 3 non-seminomas (15%). All of the *N-RAS* mutations [181C>A (Q61K)] were detected in the codon 61 of the exon 3, while the *K-RAS* mutations were detected in different codons. The most common *K-RAS* mutation [436G>A (A146T)] was in the codon 146 of the exon 4, detected in three patients. In addition, two patients had GGT>AGT (G12S) mutation in the codon 12 of the exon 2, one patient had GGC>GAC (G13D) mutation in the codon 13 of the exon 2, and another had CAA>CTA (Q61L) mutation in the codon 61 of the exon 3. In colorectal cancer, mutations in the codons 12 and 13 of the exon 2 represent the most common *K-RAS* and *N-RAS* mutations [14]. Approximately 40% of *K-RAS* gene mutations in colorectal cancer are observed in the codons 12 and 13 of the exon 2 [15]. Our results showed that the *RAS* mutations in TGCT patients are present in different codons compared to the patients with colorectal cancer.

It is important to note that the rate of *K-RAS* and *N-RAS* mutations was higher in our study compared with the other studies. This may be due to the fact that we performed extended RAS mutation testing.

In two patients, both *K-RAS* and *N-RAS* mutations were present; one patient had seminoma tumor and the other had non-seminoma tumor. The patient with seminoma had GGT>AGT (G12S) *K-RAS* mutation in the codon 12 and 181C>A (Q61K) *N-RAS* mutation in the codon 61. The patient with non-seminoma had 436G>A (A146T) *K-RAS* mutation in the codon 146 and 181C>A (Q61K) *N-RAS* mutation in the codon 61. One of these patients was treated with one cycle of carboplatin and the other with four cycles of BEP as the adjuvant therapy. No recurrence was observed in either of the two patients after the adjuvant chemotherapy.

A study by Boublikova et al. [16] revealed that there was no significant difference in the frequency of *BRAF* and *RAS* mutations between seminoma and non-seminoma patients, even though *BRAF* mutations were more common in seminomas and RAS variants were more frequent in non-seminomas [16]. Goddard et al. [17] found that testicular seminomas are associated with c-Kit mutations [17]. Skotheim et al. [18] showed gene expression differences between seminoma and non-seminoma TGCTs [18]. Feldman et al. [19] showed 4.2% rate of *K-RAS* mutations in cisplatin-resistant germ cell tumors [19]. A recent study by Honecker et al. [20] assessed 100 control (50 seminomas and 50 nonseminomatous germ cell tumors [NSGCT]) and 35 cisplatin-resistant cases of TGCTs (3 seminomas and 32 NSGCTs). A *K-RAS* mutation was not observed in the cisplatin-resistant group and was present in two cases (one seminoma and one non-seminoma; 2%) in the control group [20]. In our study, two cases were cisplatin-resistant, and these patients died before the time of analysis. Neither *K-RAS* nor *N-RAS* mutations were detected in these two cases. In addition, four recurrences were observed after orchiectomy and adjuvant chemotherapy; a *K-RAS* mutation was detected in only one of these cases (nonseminomatous type), and none of the *RAS* mutations were observed. The major limitation of our study was the small number of samples. However, this might be explained by the fact that TGCTs are uncommon tumors.

CONCLUSION

TGCTs include a heterogeneous group of tumors. To date, no approved targeted therapy is available for the treatment of TGCTs. However, analysis of *K-RAS* and *N-RAS* mutations in these tumors may provide more treatment options with a lower toxicity profile compared to the current therapies. Therefore, further studies investigating the genetic profile of TGCTs are warranted.

DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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