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ORIGINAL ARTICLE

Hotspot *TERT* promoter mutations are rare events in testicular germ cell tumors

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Abstract The abnormal activation of telomerase, codified by the telomerase reverse transcriptase (*TERT*) gene, is related to one of cancer hallmarks. Hotspot somatic mutations in the promoter region of *TERT*, specifically the c.-124:C>T and c.-146:C>T, were recently identified in a range of human cancers and have been associated with a more aggressive behavior. Testicular germ cell tumors frequently exhibit a good prognosis; however, the development of refractory disease is still a clinical challenge. In this study, we aim to evaluate for the first time the presence of the hotspot telomerase reverse transcriptase gene promoter mutations in testicular germ cell tumors. A series of 150 testicular germ cell tumor cases and four germ cell tumor cell lines were evaluated by PCR followed by direct Sanger sequencing and correlated with patient's clinical pathological features. Additionally, we genotyped the telomerase reverse transcriptase gene promoter single nucleotide polymorphism rs2853669 (T>C) located at −245 position. We observed the presence of the *TERT* promoter mutation in four patients, one exhibited the c.-124:C>T and three the c.-146:C>T. No association between *TERT* mutation status and clinicopathological features could be identified. The analysis of the rs2853669 showed that variant C was present in 22.8 % of the cases. In conclusion, we showed for the first time that *TERT* promoter mutations occur in a small subset (~3 %) of testicular germ cell tumors.

Keywords Testicular neoplasms · Neoplasms, germ cell, and embryonal · *TERT* protein · Mutation · Polymorphism, single nucleotide

Introduction

Telomere maintenance and regulation are fundamental for normal cell homeostasis, and telomerase activity is central in this process [1]. One of the key human cancer hallmarks is the abnormal upregulation of telomerase, that is codified by the telomerase reverse transcriptase (*TERT*) gene [1]. Recent

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studies identified the presence of hotspot somatic mutations in the promoter region of *TERT* gene, specifically the −124:C>T and −146:C>T mutations, in a range of human cancers such as bladder, gliomas, thyroid and melanoma [2–5]. These mutations have been shown to create a new binding motif sites for ETS transcription factors, which induces upregulation of *TERT* levels [2, 5, 6]. Recently, the less frequent variant C of the single nucleotide polymorphism (SNP) rs2853669 located −245 bp upstream from the ATG start site of the *TERT* promoter region has been shown to decrease *TERT* expression levels in both −124:C>T and −146:C>T mutant cases, apparently introducing a protective effect [7]. In accordance, this rs2853669 SNP modifies the clinical outcomes in bladder cancer and renal cell carcinoma [8, 9].

Testicular germ cell tumor (TGCT) is the most frequent malignant neoplasm in young man, representing 95 % of the testicular cancers [10]. Commonly, TGCTs are classified in two distinct groups, the seminomas (SE) and the non-seminomas (non-SE), with a slight predominance of the last group and frequently appearing as a mixed tumor containing both histologies. The mainstay TGCT treatment over the last decades has been platinum-based chemotherapy associated with surgery, leading to high response rates and also in some cases to a curable disease [11]. Nevertheless, the development of refractory disease is still a challenge in around 15 % of the cases, and even within this group, there is a large range of risk [12, 13]. Currently, the risk stratification of the patients is based only on clinical parameters as the spreading pattern and serum levels of human chorionic gonadotropin (hCG), alpha-fetoprotein (AFP), and lactate dehydrogenase (LDH).

It is well known that telomerase activity is present in normal testicular tissue and in TGCT, and its activity level has been inversely related to the differentiation state of clinical germ cell tumors [14]. Recently, a GWAS study reported an association of TGCT and genetic variants on the *TERT* locus, on chromosome 5, suggesting the role of *TERT* gene on TGCT tumorigenesis [15]. However, the molecular mechanism associated with telomerase activation in different types of germ cell tumors are complex and remain unclear. Herein, we evaluated the presence and clinicopathological impact of somatic mutations and rs2853669 polymorphism in the promoter region of *TERT* gene in a population of Brazilian and Portuguese TGCT.

Materials and methods

Human subjects and samples

Formalin-fixed paraffin-embedded (FFPE) tissues from 150 cases of testicular germ cell tumors (SE, non-SE, and mixed tumors) were retrieved from the files of the Department of Pathology at Barretos Cancer Hospital (Brazil) and Hospital de Braga (Portugal). All the patients were diagnosed between 2006 and 2012. The samples analyzed were from primary tumors at diagnosis prior the use of any possible systemic treatment. All the TGCT cases were reevaluated by a pathologist to the confirmation of the diagnosis.

Germ cell tumor cell lines

The germ cell tumor cell lines (NTERA-2, 1411H, 1777N and N2102Ep Clone2/A6) were purchased from the European Collection of Cell Cultures (ECACC). DNA was isolated from cell lines using TRIzol reagent (Life Technologies) following the manufacturer's recommendation. Authentication of cell lines was performed by short tandem repeat (STR) DNA typing according to the International Reference Standard for Authentication of Human Cell Lines, as reported by Dirks et al. [16]. Genotyping confirmed the identity of all cell lines.

DNA isolation from FPPE tissue

DNA was obtained from FFPE tissue sections representative of the tumor lesions using the QIAamp® DNA Micro Kit (Qiagen), following manufacturer's instructions and as previously described [17].

TERT mutation and SNP analysis

The analysis of hotspot mutations of *TERT* promoter regions was performed by PCR followed by direct Sanger sequencing as described previously by our group [3, 18, 19].

The electropherogram analysis of the same region allowed the genotyping of rs2853669 SNP.

Statistical analysis

To assess the relationship between variables, we used the Fisher's exact test. The overall survival was assessed by Kaplan-Meier method, and comparisons between curves were performed by log-rank test. The p value established for the statistical significance was <0.05. All the statistical analysis was performed using SPSS 19.0 software (IBM Corp, Armonk, NY, USA).

Results

Twelve samples yielded inconclusive results due to DNA quality issues. Therefore, the major clinical and pathological features of the 138 patients (70 % from Brazil and 30 % from Portugal) analyzed with conclusive results are summarized in Table 1. All IGCCCG [20] risk groups were represented, and the 5-year overall survival was 84.1 %.

Table 1 Clinicopathological and molecular features of testicular germ cell tumor (TGCT) and their association with polymorphism

Characteristics	Patients	TERT promoter mutation			SNP rs2853669				
	N (%)	c.-124:C>T	c.-146:C>T	Percent[a]	All	Carrier (C/C+C/T)	(TT)	Percent[b]	*p* value
TGCT	138	1	3	2.9	92	34	58	37	
Age (mean±SD=30 years±9.5)									
<30 years	83 (60.1)	1	2	3.6	52	17	35	32.7	0.33
≥30 years	55 (39.9)	0	1	1.8	40	17	23	42.5	
Histology group									
Non-seminoma	95 (68.8)	1	2	3.2	62	22	40	35.5	0.67
Seminoma	43 (31.2)	0	1	2.3	30	12	18	40	
Histology									
Mixed tumor	51 (37.0)	0	1	2	34	15	19	44.1	0.9
Seminoma	43 (31.2)	0	1	2.3	31	12	19	38.7	
Embryonal carcinoma	18 (13.0)	1	0	5.6	11	4	7	36.4	
Yolk sac tumor	10 (7.2)	0	1	10	6	2	4	33.3	
Immature teratoma	7 (5.1)	0	0	0	6	1	5	16.7	
Mature teratoma	4 (2.9)	0	0	0	2	0	2	0	
Choriocarcinoma	2 (1.4)	0	0	0	1	0	1	0	
Missing	3 (2.2)	0	0	0	1	0	1	0	
Serum tumor markers (AJCC)									
S0	18 (13.0)	0	0	0	17	8	9	47	0.11
S1	32 (23.2)	0	1	3.1	24	4	20	16.7	
S2	39 (28.3)	1	1	5.1	22	10	12	45.4	
S3	24 (17.4)	0	0	0	16	8	8	50	
SX	25 (18.1)	0	1	4	13	4	9	30.8	
Staging (AJCC)									
I	29 (21.0)	0	2	6.9	24	8	16	33.3	0.6
IS	22 (15.9)	0	0	0	13	7	6	53.8	
II	27 (19.6)	0	0	0	18	6	12	33.3	
III	60 (43.5)	1	1	3.3	37	13	24	35.1	
Number of metastasis site									
0	51 (37.0)	0	2	3.9	37	15	22	40.5	0.07
1	36 (26.1)	1	1	5.5	22	11	11	50	
2	26 (18.8)	0	0	0	17	2	15	11.8	
≥3	10 (7.2)	0	0	0	7	2	5	28.6	
Missing	15 (10.9)	0	0	0	9	4	5	44.4	
Chemosensitivity									
Responsive	94 (68.1)	1	0	1	68	27	41	39.7	0.78
Refractory	20 (14.5)	0	1	5	14	5	9	75.4	
No chemotherapy	24 (17.4)	0	2	8.3	10	2	8	20	
IGCCCG risk									
Good	31 (22.5)	1	0	3.2	22	5	17	22.7	0.22
Intermediate	17 (12.3)	0	1	5.9	12	7	5	58.3	
Poor	28 (20.3)	0	0	0	16	6	10	37.5	
Not applicable	51 (37.0)	0	2	3.9	37	15	22	40.5	
Missing	11 (8.0)	0	0	0	5	1	4	20	

AJCC American Joint Committee on Cancer, *TERT* telomerase reverse transcriptase, *IGCCCG* International Germ Cell Cancer Cooperative Group

[a] Percentage of *TERT* promoter mutation

[b] Percentage of carriers of the polymorphism

Table 2 *TERT* promoter mutation and clinical featuring in testicular germ cell tumors

Case	TERT mutation	SNP rs2853669	Age (year)	Histology	Primary tumor (cm)	Invasiveness[a]	Tumor marker	TNM stage	Metastasis site	IGCCCG risk	Chemosensitivity	Survival status
1	c.-124:C>T	Not analyzed	26	Embryonal carcinoma	6	+	S2	III	Lymph node	Good	Responsive	Alive
2	c.-146:C>T	Not analyzed	22	Yolk sac tumor	Unknown	−	S2	III	Lymph node	Intermediate	Refractory	Dead of disease
3	c.-146:C>T	TT	19	Mixed tumor	9	−	SX	I	n/a	n/a	n/a	Alive
4	c.-146:C>T	TT	43	Seminoma	6.5	+	S1	I	n/a	n/a	n/a	Alive

n/a not applicable

[a] Vascular invasion

The mutation screening of the hotspot promoter region of *TERT* gene (c.-124:C>T and c.-146:C>T) showed the presence of the c.-124:C>T in one patient and the c.-146:C>T *TERT* mutation in three patients with testicular germ cell tumors (Table 2 and Fig. 1). Three of the four mutated cases presented primary tumors larger than 6 cm. One of them (case 4, Table 2) was a localized pure seminoma in an older adult, and the remaining were non-SE tumors in younger patients. Only one case (case 2, Table 2) corresponded to advanced refractory disease, and the patient died due to testicular germ cell tumor. There were no significant associations between *TERT* mutation and any of the clinicopathological characteristics.

SNP rs2853669 genotyping was successful in 66.6 % of cases, and we found the following genotypes: 8.7 % C/C, 28.2 % T/C, and 63.0 % T/T. The allele frequency for T was 77.2 % and for C was 22.8 %. In the four *TERT*-mutated tumors, the SNP genotyping was possible in two and both exhibited homozygous T (Table 2).

The SNP rs2853669 (C/C+C/T) did not associate with any clinicopathological characteristic (Table 1). The 5-year overall survival of C/C+C/T versus TT was not statistically significant (respectively 94.1 versus 81.7 %, p=0.33). None of the four GCT cell lines presented mutations in the hotspot promoter regions of *TERT* gene, and concerning the rs2853669, one cell line (NTERA-2) was C/C and the remaining were T/T.

Discussion

This study reports for the first time the presence of somatic mutations in the promoter region of *TERT* gene in TGCT. We observed a frequency of 2.9 % (4/138) of *TERT* promoter mutation. Recent studies have described a high frequency of *TERT* promoter mutation in several human tumors and human cell lines [2–5, 21, 22]. In glioblastomas and papillary thyroid cancer, the mutations were associated with older age at diagnosis [3]. Another recent study demonstrated association

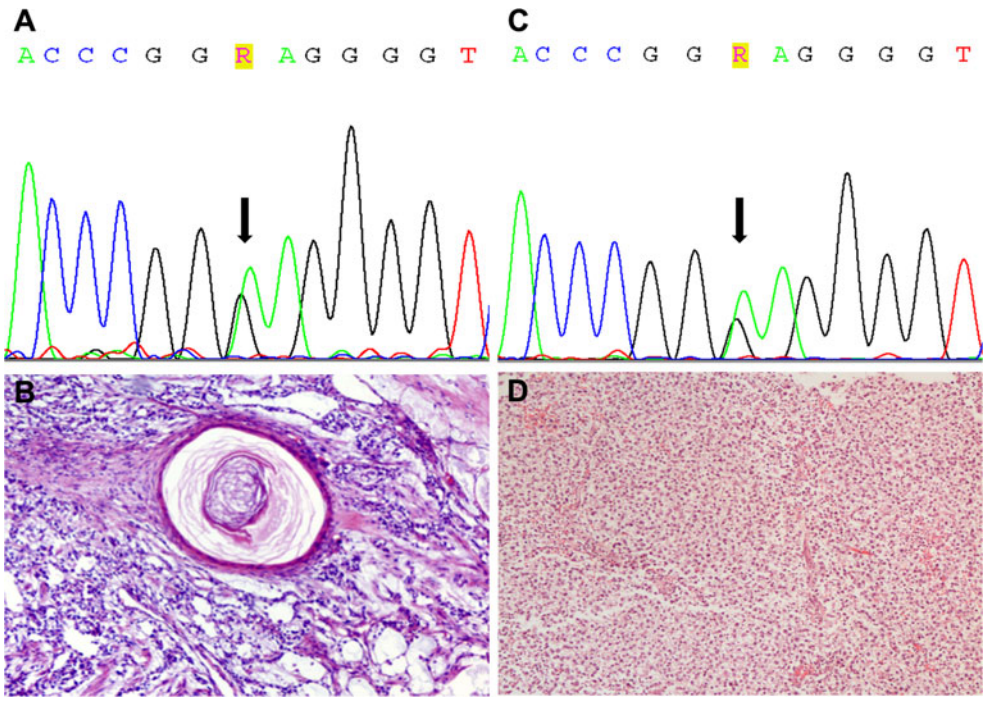

Fig. 1 Sequencing and histology of two TGCT cases presenting c.-146:C>T *TERT* promoter mutation. Case 1: **a** electropherogram reporting the c.-146:C>T mutation in a 19-year-old patient and **b** H&E staining demonstrating the mixed TGCT, containing 50 % of immature teratoma and 50 % of yolk sac tumor. Case 2: **c** electropherogram reporting the c.-146:C>T mutation in a 43-year-old patient and **d** H&E staining demonstrating the pure seminoma tissue. *Black arrow* indicates the position of c.-146:C>T mutation into the *TERT* promoter region

between *TERT* promoter mutations and older patient age, larger tumor size, distant metastases, and shorter disease specific survival in papillary thyroid cancer [23]. In melanoma, *TERT* promoter mutations were also associated with shorter disease free survival [24]. In our study, we identified *TERT* promoter mutations in small number of cases that exhibited different stages, invasiveness, and histologies, hindering meaningful statistical associations.

The *TERT* mutation profile has been associated with other molecular features in several tumor types, in particular an interesting association with *BRAF* mutation in skin melanomas and thyroid cancer [3, 4]. The frequency and clinical impact of *BRAF* mutations in TGCT is still controversial [25, 26]. *BRAF* mutations were not evaluated in the present study. It is also of note in TGCT that, at variance with the majority of the other tumor types on record, the c.-146:C>T mutation was more prevalent than the c.-124:C>T [27]. Further studies are needed to clarify if this observation represents any biological significance.

In our series, the frequency of allele C (22.8 %) of SNP rs2853669 in the *TERT* promoter region was similar to those found in more than 1000 genome database (30 %) [28]. Two of our mutated cases could not be analyzed and the other two showed T/T, the most frequent genotype. Although this polymorphism could act as a modifier of the effect of the hotspot promoter mutations on survival, as seen in bladder cancer and renal cell carcinoma [8, 9], our study has few mutated cases, hampering its statistical significance. Nevertheless, in the whole series, the carriers of the C variant for rs2853669 seem to have a better outcome. Further studies with larger cohorts would be needed to test this hypothesis.

Cell immortalization is a cancer hallmark, which is associated with abnormal telomeres size maintenance [1]. Telomere size is mainly controlled by telomerase activity, and its abnormal reactivation is reported in up to 90 % of human tumors. Only recently, Huang et al. [2] and Horn et al. [5] showed that mutations in the promoter region of *TERT* gene, namely the c.-146:C>T and the c.-124:C>T mutations, generate a new consensus binding site for ETS/TCFs transcription factors (CCGGAA) leading to a two- to fourfold increase of the *TERT* promoter activity. The low frequency of *TERT* promoter mutations observed in our study raises evidence that other pathways may be involved in telomere regulation in TGCT. It is reported that activation of telomerase can be mediated by Kit ligand in proliferating spermatogonia and primordial germ cells, but there is no telomerase activity in sperm cells [29]. Telomerase activity has not been identified in mature teratomas, while it is present in other histologies like seminoma, embryonal carcinoma, mixed tumors, as our third case, and markedly in immature teratomas [14]. In the *TERT*-mutated case, that showed a mixed (immature teratoma and yolk sac tumor—case 3, Table 2) histology, it was not possible to accurately microdissect both components, hampering to understand whether the mutation is present in both components or in just one of them. This finding fits with the mRNA expression of *TERT* [30]. Although non-SE germ cell tumors reveal longer telomeric restriction fragment than seminomas, telomerase activity seems to be similar in both groups, and other unknown factors might play a role [31]. Finally, alternative lengthening of telomeres (ATL) is a well-known mechanism involved in non-telomerase-dependent cancer cell immortalization [32], and its role in TGCT is unexplored.

Our series is very heterogeneous, representative of several types of germ cell tumor histologies, clinical staging, and response to chemotherapy. The predominance of advanced disease and non-SE histologies in our series differs from the classical literature, where seminomas and stage I disease is the most common finding [33]. Our hospital is a reference center for TGCT, associated to the Brazilian Childhood Germ Cell Tumor Study Group, a consortium developed to standardize diagnostic assessment and multidisciplinary treatment of TGCT patients in Brazil [34] and for that reason, our series might be biased by more advanced cases. However, the several risk groups were well represented in the study.

Conclusions

TERT promoter mutations seem to be a rare event in TCGTs; however, we showed for the first time that *TERT* promoter mutations can be a new molecular event in a small subset (2.9 %) of cases. Further studies are needed to extend and validate these findings, in order to assess the clinical impact of *TERT* mutations and rs2853669 polymorphism in TGCT patients and to associate *TERT* with the other molecular features of TGCT, in particular with *BRAF* mutation *status*.

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Compliance with ethical standards

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Conflicts of interest None

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee (register number CAAE 12297713.0.0000.5437) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. For this type of study, formal consent is not required.

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