

Low mutation percentage of *KRAS* and *BRAF* genes in Brazilian anal tumors

LUCAS TADEU BIDINOTTO^{1,2}, CARLOS A R VÉO¹, EDGAR ALEMAN LOAIZA¹,
ALESSANDRA PAULINO SANTOS DE FRANÇA¹, ADRIANA TARLA LORENZI¹, LUCIANA ALBINA REIS ROSA³,
CRISTINA MENDES DE OLIVEIRA³, JOSÉ EDUARDO LEVI³, CRISTOVAM SCAPULATEMPO-NETO^{1,4},
ADHEMAR LONGATTO-FILHO^{1,5,6} and RUI MANUEL REIS^{1,5,6}

¹Molecular Oncology Research Center, Barretos Cancer Hospital, Barretos, São Paulo 14784 400;

²Barretos School of Health Sciences, Dr. Paulo Prata-FACISB, Barretos, São Paulo 14785-002;

³Laboratory of Virology, Institute of Tropical Medicine, University of São Paulo, São Paulo 05403 000;

⁴Department of Pathology, Barretos Cancer Hospital, Barretos, São Paulo 14784 400, Brazil;

⁵Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho,

Braga 4710-057; ⁶ICVS/3B's-PT Government Associate Laboratory, Braga 4710-057, Portugal

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Abstract. Anal cancer is a rare type of digestive tract disease, which has had a crescent incidence in a number of regions. Carcinomas are most frequently found, with squamous cell carcinoma (SCC) comprising ~95% of all anal tumors. The major risk factor for development of this type of tumor is human papillomavirus (HPV) infection. However, previous studies have identified patients with anal cancer that are HPV-/p16- and observed that they have a poorer outcome compared with HPV+/p16+ patients. This suggests that molecular profile may drive anal cancer progression. The aim of the present study was to evaluate the mutational status of two important oncogenes, *KRAS* and *BRAF*, in a series of anal cancer lesions. Resected tumors of the anal canal (n=43) were evaluated, nine of these were high-grade squamous intra-epithelial lesion cases (HSIL), 11 were adenocarcinomas, and 23 SCCs. Direct sequencing of *KRAS* proto-oncogene, GTPase (*KRAS*; codons 12 and 13) and B-Raf proto-oncogene, serine/threonine kinase (*BRAF*; codon 600) was performed and associated with patient clinicopathological and molecular features. There was a trend of poorer prognosis of adenocarcinoma compared with HSIL and SCC. Analysis indicated one SCC patient (2.3%) exhibited a *KRAS* p.G13D mutation, and one adenocarcinoma patient (2.3%) exhibited a *BRAF* p.V600E mutation. It was observed that, these mutations are rare in anal tumors, and certain patients

may be at a disadvantage using targeted therapies based on *KRAS* and *BRAF* mutational status. As there is a low mutation percentage in SCCs, adenocarcinomas and HSIL, there may exist other underlying molecular alterations that result in anal cancer development, which require further elucidation.

Introduction

Anal cancer is a rare type of digestive tract disease, which has had an increasing incidence in a number of regions (1-3). It is estimated a total of >7,200 new cases were diagnosed in the United States in 2015, with ~1,000 anal cancer-associated mortalities (4). Tumors in this site are classified, according to World Health Organization (WHO), as intraepithelial neoplasias, carcinomas and carcinoid tumors (5). Carcinomas are most frequently identified, with squamous cell carcinoma (SCC) comprising ~95% of all the anal tumors (6), and ~5% of the lesions are adenocarcinomas (7). The age-standardized incidence is <1/100,000 people, and the mortality is 0.2/100,000 (1). In men who practice anal receptive intercourse, the incidence of anal cancer increases up to 35/100,000 (5,8). This is predominantly due to increased risk of human papillomavirus (HPV) infection (1), HPV16 is most frequently observed in anal SCC (9). HPV infection leads to intraepithelial neoplasia that progresses from low-grade to high-grade dysplasia and, finally, to invasive cancer. The regression of high-grade dysplastic lesions is rare (5). HPV infection results in high expression of cyclin-dependent kinase inhibitor 2A (p16), and disrupts the association between retinoblastoma protein and the E2F family of transcription factors, ultimately leading to cellular proliferation (10). Recently, it was demonstrated that a high frequency of women with cervical cancer also have infection of the anal canal by HPV16 (11). In addition to HPV infection, other known risk factors of anal cancer are immunodeficiency due to human immunodeficiency virus seropositivity, low cluster of differentiation 4 T cell count,

Correspondence to: Dr Rui Manuel Reis, Molecular Oncology Research Center, Barretos Cancer Hospital, 1331 Rua Antenor Duarte Villela, Barretos, São Paulo 14784 400, Brazil
E-mail: ruireis.hcb@gmail.com

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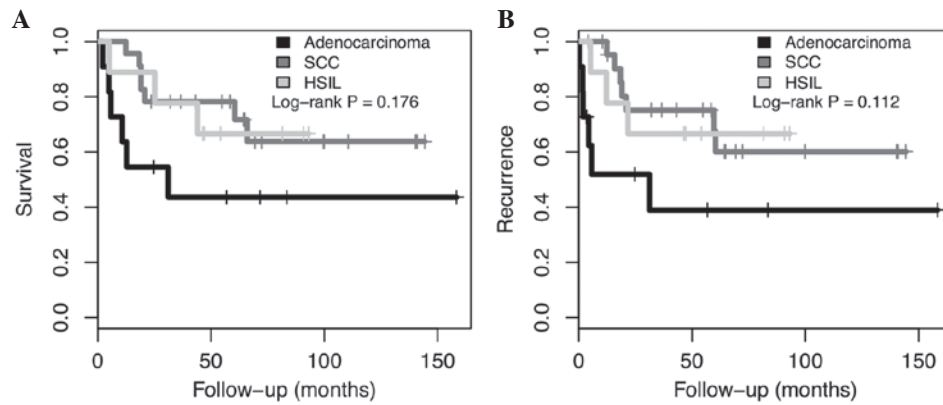


Figure 1. Kaplan-Meier curves representing (A) overall survival and (B) disease-free survival of HSIL, adenocarcinoma and squamous cell carcinoma patients. HSIL, high-grade squamous intra-epithelial lesion.

immunosuppression following solid organ transplantation, and tobacco smoking (1,5).

Previous studies have indicated that patients presenting with no HPV infection and no p16 expression (HPV-/p16-) have a poorer outcome than patients that are HPV+/p16+, and suggest an optimization in the therapy to the former (12,13), and that improved molecular characterization should be performed. A previous study demonstrated that patients with SCC of the anal canal and HPV were irresponsive to standard chemoradiotherapy treatment and frequently presented with *TP53* mutations (13). Furthermore, an additional study using immunohistochemistry (IHC), fluorescence *in situ* hybridization and next generation sequencing, observed alterations in molecular markers that may aid in the understanding of failure of certain therapeutic strategies, including overexpression of multidrug resistance-associated protein 1, DNA excision repair protein ERCC-1 and thymidylate synthetase, and suggest potential therapeutic targets, including the tyrosine kinase receptor, epidermal growth factor receptor (EGFR) (14). Molecular therapies targeting EGFR, such as cetuximab and panitumumab are currently used in colorectal cancer treatment, and tumor genetic make-up, including mutational status of *KRAS* and *BRAF* may predict patient response (15).

The aim of the present study is to evaluate the mutational status of two important oncogenes, *KRAS* and *BRAF* in a series of SCC, adenocarcinomas and high-grade squamous intra-epithelial lesions (HSILs), and whether they are associated with patient's clinicopathological features, and HPV status.

Materials and methods

In the current study, resected tumors of the anal canal from 43 patients were evaluated. The present study was approved by the ethics committee of Barretos Cancer Hospital (Barretos, Brazil) and informed consent was obtained from each patient. Histological review of the slides was performed by an expert pathologist (Dr Cristovam Scapulatempo-Neto), who confirmed the diagnosis and delimited the area of the slide containing the neoplastic lesion. Clinical data of the patients was obtained, and is summarized in Table I. HPV16 and HPV18 status, and immunohistochemical analysis of β -globin, p16, antigen Ki67 (Ki67), minichromosome maintenance protein

complex (MCM) and DNA topoisomerase 2- α (TOP2A) were retrieved from a previous study (16).

The histological slides (10 μ m) were processed, and DNA was isolated from macrodissected tumor area of one unstained section as previously described (17). The slides were placed at 80°C for deparaffinization for 10 min and hydrated with xylene and graded ethanol (100, 70 and 50%). DNA was isolated using QIAamp DNA Micro kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's protocols, and quantified using NanoDrop 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The samples were diluted to a final concentration of 50 ng/ μ l and stored at -20°C for further analysis.

The hotspots of *KRAS* (codons 12 and 13) and *BRAF* (codon 600) were amplified using PCR and sequenced, as previously described (17). Amplification of *KRAS* was performed in a final reaction volume of 15 μ l containing 1.5 μ l buffer (Qiagen GmbH), 2 mM MgCl₂ (Qiagen GmbH), 100 mM dNTPs (Invitrogen; Thermo Fisher Scientific, Inc.), 0.2 mM sense and 0.2 mM anti-sense primers (Sigma-Aldrich, St. Louis, MO, USA), 1 unit HotStarTaq DNA polymerase (Qiagen GmbH) and 1 μ l DNA. The *KRAS* region was amplified using the following primers: Sense, 5'-GTGTGACATGTTCTAATATAGTCA-3' and anti-sense, 5'-GAATGGTCCTGCACCAGTAA-3'. The *BRAF* amplification reaction was performed as described above, with 0.3 mM sense and anti-sense primers used. The region was amplified using the following primers: Sense, 5'-TCATAATGCTTGCTCTGATAGGA-3' and anti-sense, 5'-GGCCAAAATTAAATCAGTGGA-3'. The following cycling conditions were used: Initial denaturation at 96°C for 15 min, followed by 40 cycles of denaturation at 96°C for 45 sec, annealing at 55.5°C for 45 sec, then extension at 72°C for 10 min, all using a Veriti 96-Well thermal cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.)

The PCR products were purified with EXO-SAP (GE Healthcare Life Sciences, Chalfont, UK), and sequenced using 1 μ l BigDye (Applied Biosystems; Thermo Fisher Scientific, Inc.), 1.5 μ l sequencing buffer (Applied Biosystems; Thermo Fisher Scientific, Inc.) and 1 μ l primer (Thermo Fisher Scientific, Inc.). The sequencing reaction, which consisted of 30 cycles of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec and extension at 60°C for 4 min, was followed by post-sequencing purification with EDTA, alcohol and sodium citrate. The products of PCR were eluted in Hi-Di formamide (Thermo Fisher

Table I. Clinicopathological features of patients with anal lesions.

Parameter	Frequency (%)		
	HSIL	Adenocarcinoma	SCC
Gender			
Female	6 (20.7)	7 (24.1)	16 (55.2)
Male	3 (21.4)	4 (28.6)	7 (50.0)
Ethnicity			
Caucasian	8 (21.6)	8 (21.6)	21 (56.8)
Non-caucasian	1 (16.7)	3 (50)	2 (33.3)
History of previous disease			
No	2 (11.1)	7 (38.9)	9 (50.0)
Yes	7 (29.2)	3 (12.5)	14 (58.3)
NA	0	1 (100)	0
History of tumor in the family			
No	6 (21.4)	6 (21.4)	16 (57.1)
Yes	3 (21.4)	4 (28.6)	7 (50.0)
NA	0	1 (100)	0
Tobacco consumption			
No	1 (5.6)	7 (38.9)	10 (55.6)
Yes	7 (33.3)	3 (14.3)	11 (52.4)
NA	1 (25.0)	1 (25.0)	2 (50.0)
Surgery			
No	6 (26.1)	6 (26.1)	11 (47.8)
Yes	3 (16.7)	5 (27.8)	10 (55.6)
NA	0	0	2 (100)
Radiotherapy			
No	2 (40.0)	1 (20.0)	2 (40.0)
Yes	7 (19.4)	10 (27.8)	19 (52.8)
NA	0	0	2 (100)
Chemotherapy			
No	2 (25.0)	3 (37.5)	3 (37.5)
Yes	7 (21.2)	8 (24.2)	18 (54.5)
NA	0	0	2 (100)
Response to the treatment			
No response	4 (23.5)	6 (35.3)	7 (41.2)
Complete response	1 (8.3)	2 (16.7)	9 (75.0)
Progression	2 (22.2)	2 (22.2)	5 (55.6)
NA	2 (40.0)	1 (20.0)	2 (40.0)
Recurrence			
No	7 (22.6)	6 (19.4)	18 (58.1)
Yes	2 (16.7)	5 (41.7)	5 (41.7)
Status			
Death by cancer	3 (23.1)	4 (30.8)	6 (46.2)
Death by other cause	0	2 (66.7)	1 (33.3)
Alive, free of disease	6 (27.3)	3 (13.6)	13 (59.1)
Alive, with the disease	0	2 (40.0)	3 (60.0)
Age (years)			
<48	3 (33.3)	1 (11.1)	5 (55.6)
48-66	5 (20.0)	6 (24.0)	14 (56.0)
>66	1 (11.1)	4 (44.4)	4 (44.4)

NA, not available.

Table II. Molecular features of anal lesion patients.

Histological type	Gender	Age (years)	Immunohistochemistry ^a				Mutation		Treatment	Oncological response	DFS (months)	Survival (months)	Status		
			HPV16 ^a	HPV18 ^a	β-globin	p16	K167	MCM						TOP2A	KRAS
HSIL	M	50	+	-	Ok	+	4+	3+	wt	wt	Surgery + RDT + QT	Progression	21.74	25.10	D
HSIL	F	57	+	-	Weak	+	4+	2+	wt	wt	RDT + QT	No evidence	47.17	47.17	A
HSIL	F	57	+	-	Ok	+	4+	3+	wt	wt	RDT + QT	No evidence	5.13	5.13	D
HSIL	F	47	+	-	Ok	+	4+	1+	wt	wt	Surgery + RDT + QT	Progression	12.20	44.05	D
HSIL	M	38	+	+	Ok	+	4+	3+	wt	wt	RDT + QT	No evidence	54.11	54.11	A
HSIL	F	64	+	-	Ok	-	4+	2+	wt	wt	No treatment	No evidence	46.38	46.38	A
HSIL	F	69	+	-	Ok	+	4+	-	wt	wt	RDT + QT	NA	93.03	93.03	A
HSIL	F	53	+	-	Ok	+	4+	1+	wt	wt	RDT + QT	NA	90.79	90.79	A
HSIL	M	27	+	-	Ok	-	4+	2+	wt	wt	Surgery	CR	81.51	81.51	A
ADC	F	35	+	-	Ok	-	4+	-	wt	wt	Surgery + RDT + QT	Progression	1.74	12.86	D
ADC	M	69	+	-	Ok	+	4+	-	wt	wt	RDT + QT	No evidence	4.38	10.82	D
ADC	M	66	-	-	Ok	-	NA	1+	wt	wt	RDT	No evidence	1.88	4.97	D
ADC	F	86	+	-	Ok	-	3+	-	wt	wt	RDT	No evidence	31.15	31.15	D
ADC	F	60	+	-	Ok	-	4+	-	wt	wt	RDT + QT	No evidence	5.76	5.76	D
ADC	M	70	+	-	Ok	+	4+	3+	wt	V600E	Surgery	No evidence	0.13	2.11	D
ADC	F	59	+	-	Ok	+	4+	3+	wt	wt	RDT + QT	NA	56.78	56.78	A
ADC	M	58	+	-	Ok	+	1+	+CB	wt	wt	Surgery + RDT + QT	CR	24.70	24.70	A
ADC	F	63	+	-	Ok	-	4+	-	wt	wt	RDT + QT	Progression	3.42	71.61	A
ADC	F	52	-	-	Ok	-	4+	1+	wt	wt	Surgery + RDT + Q	No evidence	83.42	83.42	A
ADC	F	81	+	-	Ok	-	2+	+CB	wt	wt	Surgery + RDT + QT	CR	158.36	158.36	A
SCC	M	85	+	-	Ok	+	4+	3+	wt	wt	Surgery + RDT	No evidence	19.05	19.05	D
SCC	M	64	+	-	Ok	+	3+	3+	wt	wt	Surgery + RDT + QT	No evidence	59.64	65.86	D
SCC	M	50	+	-	Ok	NA	4+	2+	wt	wt	RDT + QT	Progression	4.14	23.49	A
SCC	F	43	+	-	Weak	+	4+	-	wt	wt	RDT + QT	Progression	58.42	58.42	A
SCC	M	43	+	-	Ok	-	1+	-	wt	wt	Surgery + RDT + QT	Progression	31.97	31.97	A

Table II. Continued.

Histological type	Gender	Age (years)	Immunohistochemistry ^a										Mutation		Treatment	Oncological response	DFS (months)	Survival (months)	Status
			HPV16 ^a	HPV18 ^a	β-globin	p16	Ki67	MCM	TOP2A	KRAS	BRAF	KRAS	BRAF						
SCC	F	44	+	-	Ok	+	4+	3+	3+	3+	3+	wt	wt	NA	CR	36.61	36.61	A	
SCC	F	48	+	-	Ok	+	4+	2+	3+	3+	3+	wt	wt	RDT + QT	No evidence	18.36	18.36	D	
SCC	F	62	+	-	Ok	+	4+	1+	2+	2+	2+	wt	wt	Surgery + RDT + QT	Progression	15.86	19.05	D	
SCC	F	56	+	-	Ok	+	4+	3+	2+	3+	2+	wt	wt	RDT + QT	CR	64.34	64.34	A	
SCC	F	64	+	-	Weak	+	4+	3+	3+	3+	3+	wt	wt	Surgery + QT	No evidence	60.39	60.39	D	
SCC	F	68	-	-	Ok	+	4+	2+	2+	2+	2+	wt	wt	Surgery + RDT + QT	No evidence	12.37	12.37	D	
SCC	F	58	+	-	Ok	+	4+	3+	2+	3+	2+	wt	wt	NA	NA	69.31	69.31	A	
SCC	M	56	+	-	Ok	+	4+	2+	NA	2+	NA	wt	wt	RDT + QT	No evidence	12.70	99.61	A	
SCC	M	34	-	-	Ok	+	4+	3+	3+	3+	3+	wt	wt	RDT + QT	CR	54.77	54.77	A	
SCC	F	44	+	-	Ok	NA	2+	1+	1+	1+	1+	wt	wt	RDT + QT	NA	43.16	43.16	A	
SCC	F	48	+	-	Ok	-	3+	2+	1+	2+	1+	wt	wt	No treatment	No evidence	20.82	20.82	D	
SCC	F	56	+	-	Ok	+	4+	2+	2+	2+	2+	wt	wt	Surgery + RDT + QT	CR	64.80	64.80	A	
SCC	F	79	+	-	Ok	+	4+	3+	2+	3+	2+	wt	wt	RDT + QT	CR	72.27	72.27	A	
SCC	F	60	+	-	Ok	+	4+	4+	2+	2+	2+	wt	wt	Surgery + RDT	Progression	10.46	110.53	A	
SCC	M	57	+	-	Ok	+	4+	2+	3+	2+	3+	wt	wt	Surgery + RDT + QT	CR	144.31	144.31	A	
SCC	F	57	+	-	Weak	+	3+	2+	2+	2+	2+	wt	wt	RDT + QT	CR	140.95	140.95	A	
SCC	F	58	+	-	Ok	+	4+	-	NA	-	NA	G13D	wt	Surgery + RDT + QT	CR	140.23	140.23	A	
SCC	F	80	+	-	Ok	+	4+	-	2+	-	2+	wt	wt	RDT + QT	CR	99.93	99.93	A	

^aAs described in Scapulatempo-Neto *et al* (16). HSIL, high-grade squamous intra-epithelial lesion; ADC, adenocarcinoma; SCC, squamous cell carcinoma; F, female; M, male; HPV, human papillomavirus; p16, cyclin-dependent kinase inhibitor 2A; Ki67, antigen Ki67; MCM, minichromosome maintenance protein complex; TOP2A, DNA topoisomerase 2-α; KRAS, KRAS proto-oncogene, GTPase; BRAF, B-Raf proto-oncogene, serine/threonine kinase; wt, wild type; RDT, radiotherapy; QT, chemotherapy; CR, complete response; DFS, disease-free survival; D, deceased; A, alive.

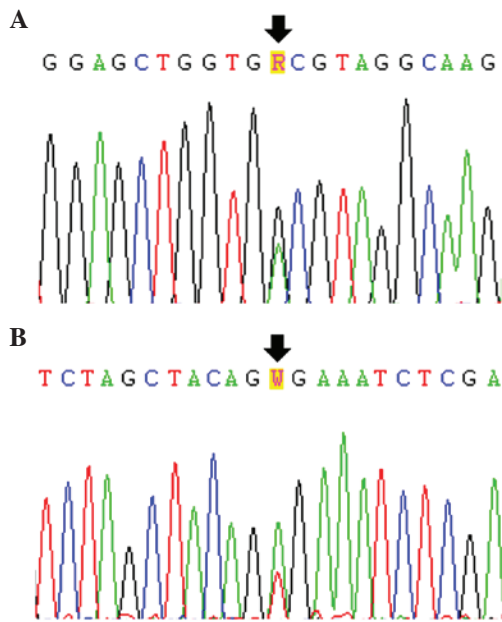


Figure 2. Electropherograms representing the mutations in (A) *KRAS* (p.G13D) and (B) *BRAF* (p.V600E).

Scientific, Inc.) and incubated at 95°C for 5 min and at -4°C for at least 5 min. Direct sequencing was performed in a 3500 Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). The mutations were confirmed with two independent reactions.

Survival analysis was performed considering the three different histology types using Kaplan-Meier plots and log rank statistical analysis using R.

Results

Histological review of the slides demonstrated that, from the 43 patients, 9 were diagnosed with HSIL, 11 patients were diagnosed with adenocarcinomas, and 23 with SCC. The mean age at diagnosis varied from 51 (HSIL) to 64 (adenocarcinoma) years. Overall survival (OS) and disease-free survival (DFS), based on the histologic type is presented in Fig. 1. There was a trend of poorer prognosis in adenocarcinoma compared with HSIL and SCC ($P=0.176$ and $P=0.112$ for OS and DFS, respectively).

Of the 43 patient samples examined, 1 (2.3%), exhibited a *KRAS* mutation, which was p.G13D (Fig. 2A and Table II). This case was a female SCC patient (age, 58) with previous gynecological or anal disease. The SCC was positive for β -globin and p16 using IHC, as well as 4+ Ki67 labeling. Notably, this patient also presented with HPV16 anal infection (Table II). Following surgery, radiotherapy and chemotherapy treatment, the patient exhibited complete response, and was free of disease at the last follow up of 140 months (Table II).

A *BRAF* mutation, p.V600E, was also observed in only 1/43 patients (2.3%; Fig. 2B and Table II). This case was an adenocarcinoma of a male 70 year-old tobacco smoking patient. Similarly to the case described above, the patient also presented with HPV16 anal infection, and IHC indicated positivity for β -globin and p16 IHC labeling, as well as 4+ Ki67 labeling (Table II). In addition, the sample presented 3+ MCM and 3+ TOP2A IHC labeling. The patient relapsed and succumbed to the condition 2 months following surgery (Table II).

Discussion

The present study aimed to evaluate the mutational status of *KRAS* and *BRAF* in tumors arising in the anal canal, and to associate these findings with clinicopathological data. To the best of our knowledge, the majority of the mutational screening of *KRAS* and *BRAF* in anal tumors focused on SCC samples due to the predominance of this histological type in anal tumors.

Although tumors located in the anal region may be anatomically close to colorectal tumors, they exhibit different histological patterns, distinct features, and therefore distinct etiologies (18). Previous studies have observed a high incidence of *KRAS* mutation in colorectal cancer (CRC) worldwide (19), and in Brazilian populations (17,20). Overall, among 8,234 Brazilian CRC cases analyzed, the *KRAS* mutation frequency of 31.9%, with the majority of these samples exhibiting a p.G12D mutation (20). *KRAS* mutations generally arise in codons 12 or 13, and constitutively activate its pathway. The protein generated by the mutated gene is capable of transmitting the signal independently of tyrosine kinase receptor activation (21). Of the 43 patients analyzed, 1 patient with SCC was observed to have a *KRAS* mutation (2.3%). This low mutation rate is consistent with previous studies, which identified no *KRAS* mutation in SCC of the anal canal samples [$n=36$ samples (22), $n=89$ samples (23), $n=53$ samples (24) and $n=66$ samples (25)]. Additional studies observed a total of 1.6% ($n=193$ samples) (26), and 5% ($n=84$ samples) (27) of SCC to have a *KRAS* mutation. Furthermore, *BRAF*, another mitogen-activated protein kinase pathway gene, which was identified as mutated in ~50% of melanomas (28), presented a low mutation rate in the samples in the present study (2.3%). This gene was observed with a low percentage of mutation in anal tumors, varying from 0% (24,25,27) to 4.7% (26) consistent with the findings of the current study. In addition, this gene was also found mutated in a low percentage of precursor lesions of colorectal cancer (17) and colorectal cancer (29). It is important to highlight that no mutation was observed in patients with HSIL, which suggest that these mutations may occur preferentially in tumors with an invasive phenotype.

The standard treatment of anal SCC in the majority of patients is chemotherapy and radiotherapy, with a response rate of up to 80% (30). Metastatic and refractory cases are rare, although, it has been demonstrated that cetuximab-based treatment results in disease progression of *KRAS*-mutated tumors, while those with wild type *KRAS* exhibited partial or minor remission (31). This data is consistent with further studies in colorectal cancer that demonstrated *KRAS* and *BRAF* mutational status predicted tumor response to targeted therapies (15). Thus, an understanding of *KRAS* and *BRAF* mutational status is key in personalized medicine.

Using the mutational rate of *KRAS* and *BRAF*, the present study evaluated the anatomical association between anal and rectal tumors, tumors arising in the anus have different *KRAS* mutation percentage of the rectal counterparts, thus, the molecular differences require elucidation. To the best of our knowledge, the current study is the first to evaluate the percentage of *KRAS* and *BRAF* mutations in adenocarcinomas and HSIL of the anal canal, in addition to SCC. Furthermore, to date, the present study is the first to describe these mutations in tumors of anal canal of the Brazilian population. In addition

to the well-known risk factor of HPV infection that drives anal cancer tumorigenesis, there are patients who develop these tumors in the absence of this infection. The present study evaluated the mutation percentage of two well-known drivers of colorectal cancer (*KRAS*) and melanoma (*BRAF*) to further elucidate other risk factors in anal cancer development. In conclusion, a low percentage of mutation was identified in SCCs, adenocarcinomas and HSIL, however, these tumors may exhibit other molecular alterations that result in anal cancer development, which require elucidation in future studies.

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