Original Article

ASSESSMENT OF *TP53, PTEN* AND *AKT* GENE MUTATIONS IN TONGUE SQUAMOUS CELL CARCINOMA AND ORAL LEUKOPLAKIA OF SMOKERS AND NON-SMOKERS

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ABSTRACT

Oral Squamous Cell Carcinoma (OSCC) is the most common malignant tumor of the
oral cavity. It can be preceded by Oral leukoplakia (OL), the most prevalent potentially
malignant disorder, which presents a malignant transformation rate which varies from 1,1%
to 40,8%, found most commonly in tongue lesions. Smoking is a well-established risk factor
for both OL and OSCC. In this study we aimed to investigate loss of heterozygosity (LOH) as
well as the frequency and patterns of the mutations in the *PTEN, AKT* and *TP53* genes in OL
and tongue squamous cell carcinoma (TSCC) samples of smokers and non-smokers patients.
Tissue specimens were obtained from 73 patients: 35 cases of TSCC and 38 cases of high
grade OL occurring in the lateral border of the tongue, and *PTEN, AKT* e *TP53* gene
mutations were investigated through Fluorescence In Situ Hybridization and genotyping.
Differences between the genetic changes studied in the TP53, PTEN and AKT genes in
smoking and non-smoking patients were detected. Changes in the *TP53* gene did not occur
significantly in the OL stage in either smoking or non-smoking patients, whereas changes in
the *PTEN* gene were observed in both OL and OSCC, with a statistically significant
difference between smokers and non-smokers. Furthermore, changes in the AKT gene did not
occur with high frequency in either OL or carcinomas, regardless of smoking status,

suggesting that disruptions in the critical PI3K pathway are primarily due to genetic changes in PTEN rather than in the AKT gene.

Statement of Clinical Significance

The present study highlights the importance of early diagnosis of oral cancer, as demonstrated in the study, we present significant alterations that can occur in both early and late stages. Mutations and translocations provide early indications that could lead to a better understanding of oral cancer development, potentially paving the way for new research lines and increasing the chances of early identification.

INTRODUCTION

Oral Squamous Cell Carcinoma (OSCC) is the most common malignant tumor of the oral cavity [1]. A significant number of OSCC can be preceded by asymptomatic clinical lesions collectively referred to as oral potentially malignant disorders (OPMD). Oral leukoplakia (OL) is the most prevalent OPMD, with a global prevalence of 2–3%. The reported rate of OL malignant transformation varies from 1,1% a 40,8%, with tongue lesions being the most commonly affected site[2].

Oral carcinogenesis is a multistep process and a combination of environmental risk factors, viral infection, genetic and epigenetic alterations might give rise to OSCC [7]. In this sense, several studies have focused on molecular alterations of OL in an attempt to identify predictive biomarkers for malignant transformation. Several molecular markers, used alone or in combination, have been recognized as important in the study of oral carcinogenesis [8]. However, the frequency of mutation in genes like *PTEN (*a tumor suppressor gene, which

frequently shows loss of heterozygosity (LOH) or mutations in human cancer), *AKT (*PTEN's encoded protein which controls critical cellular pathways, including those leading to apoptosis inhibition and increased cell proliferation) and *TP53 (*a tumor suppressor gene and it is the most frequently altered gene in human cancer genes) in both OL and OSCC remains to be investigated. Additionally, it is not clear if such frequencies are similar in smokers and non-smokers.

Therefore, in this study, we aimed to investigate LOH, as well as the frequency and patterns of the mutations in the *PTEN*, *AKT,* and *TP53,* a tumor suppressor gene and it is the most frequently altered gene in human cancer genes in OL and tongue squamous cell carcinoma (TSCC) samples of smokers and non-smokers patients.

MATERIALS AND METHODS

Tissue samples

The Formalin-fixed, paraffin-embedded (FFPE) tissue specimens were obtained from 73 patients: 35 cases of TSCC and 38 cases of OL with high grade dysplasia occurring in the lateral border of the tongue. Histological sections (5 µm) of all samples were routinely stained with haematoxylin and eosin (H&E) and analyzed under light microscopy. Two independent oral pathologists without prior knowledge of the clinical data assessed the stained sections to classify the histological grades of oral dysplasia according to previously proposed binary system criteria of classification [9] . Despite the use of the binary system, it is important to note that additional histological features, such as sharp lateral margins, have been recognized as important in dysplasia grading, even though they are not included in the standardized grading criteria yet [10]. In the cases of disagreement, pathologists discussed the findings to achieve a final agreement.

Ethical aspects

This study was approved by the Ethical Committee of the Piracicaba Dental School, University of Campinas, Piracicaba, Brazil (process number 6.010.777).

Nucleic acid extraction

Eight 4-micrometer sections of formalin-fixed, paraffin-embedded (FFPE) extracted tumor material were incubated in ATL buffer at 56°C for 48 h, with an additional 20 µl of Proteinase K (20 mg/ml). Genomic DNA (gDNA) was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany), following the manufacturer's instructions. The DNA was eluted twice in ATE buffer to reach a final elution volume of 20 µl. DNA quality (260/280 and 260/230 ratios) and quantity (concentration) were determined using a Nanodrop 2000 spectrometer (Thermo Scientific). The size profile of the extracted genomic DNA was determined by 1.5% agarose gel electrophoresis.

PTEN, AKT and TP53 copy number

FISH was used to evaluate *PTEN*, *TP53* and *AKT* copy number in a subset of 73 samples. FISH was performed according to the protocol of Pinkel et al. [10] with modifications introduced by Calcagno et al [11]. Cells were hybridized with Spectrum Orange Probe (LSI Vysis/Abbott, Inc., IL) and nuclei were counterstained with 49,6-diamidino-2-phenylindole antifade. Fluorescence was detected using an Olympus BX41 fluorescence microscope (Olympus, Japan) with excitation filters for 49,6- diamidino-2-phenylindole (260 nm) and rhodamine (570 mn). For each case, 200 interphase nuclei were analyzed using an ASI image analysis system (Applied Spectral Imaging, Israel). Positive *PTEN*, *TP53* and *AKT* gene signals appeared as red spots in nuclei and were scored using the criteria of Hopman et al. [12]

To avoid misinterpretation due to technical error, normal lymphocyte nuclei and normal oral tissue were used as controls. The FISH results were presented as the percentage of *PTEN, TP53* and *AKT* amplification by a cell, in which the percentage of cells showing 3 or more signals for *PTEN, TP53* and *AKT* probes by cell were calculated.

PTEN, AKT and TP53 genotyping

Exons of *PTEN, TP53* and *AKT* genes were selected for mutation analysis in all 73 OSCC/OL samples. The PCR reactions were carried out with 0.1 mmol/L of dNTPs, 2 mmol/L of MgCl2, 0.5 mmol/L of primers, 1 U of Taq polymerase, and 100 ng of DNA. The primers sequences were: PTEN forward primer 5'-TGAGTTCCCTCAGCCGTTACCT - reverse primer 5'-GAGGTTTCCTCTGGTCCTGGTA, TP53 forward primer 5'-CCTCAGCATCTTATCCGAGTGG- reverse primer 5'-TGGATGGTGGTACAGTCAGAGC, AKT forward primer 5'-TGGACTACCTGCACTCGGAGAA - reverse primer 5'-GTGCCGCAAAAGGTCTTCATGG). The PCR reactions followed standard conditions. The amplicons were separated on a 2% agarose gel stained with SYBRH Safe DNA Gel Stain (Life Technolgies, USA) and directly visualized under UV illumination.

Amplicons were sequenced using the Sanger method. Direct sequencing was carried out using the Big DyeH Terminatorv3.1 Cycle Sequencing kit (Life Technologies, USA) and analyzed on an ABI PRISMH 3130 Genetic Analyzer (Life Technologies, USA) using Pop 7 polymer.

The sequencing chromatograms were inspected using the Chromas Pro 1.5 (Technelysium Pty Ltd, Australia). The reference sequences were Gene ID: 5728 (NCBI), ID: 7157 (NCBI), ID: 207 (NCBI) . Variants with less than 1% minor allele frequency were reported. The predicted pathogenicity of missense mutations was assessed by *in silico*

analysis using PolyPhen (http://genetics.bwh.harvard.edu/ pph/) and SIFT (http://sift.jcvi.org).

Statistical analysis

To determine any significant differences in allelic loss and mutation frequency of *PTEN, TP53* and *AKT* among the groups investigated, a Fisher's Exact test was applied. Statistical analysis was conducted using RStudio Team 2023 (RStudio Team, Boston, MA, USA), with a significance level set at $p < 0.05$ and 95% confidence intervals.

RESULTS

Sample characterization

This study included 35 patients (20 male and 15 female) with TSCC. Mean age of 63.5 years was observed, ranging from 27 to 100 year-old. Eighteen (51.4%) were smokers, and 17 (48.6%) were non-smokers. Regarding the OL cases, 38 (11 male and 27 female) high grade cases in the lateral border of the tongue were retrieved. Seventeen (44.7%) were smokers and 21(55.3%) were non-smokers. Mean age of 57.5 years was observed, ranging from 32 to 83 year-old.

Tongue squamous cell carcinoma molecular results

In tobacco-associated OSCC, *TP53* LOH was detected in 14/18 (77.8%) of the cases. The most frequent mutation was missense (12/15 - 85.7%), followed by frameshift deletion (2/14 - 14.3%). There was a case of LOH with no mutation in the remaining allele.

In non-smokers, *TP53* LOH was detected in 11/17 (52.9%) of the cases. The most frequent mutation was missense (6/11 - 66.7%), followed by frameshift deletion (2/11 - 22.2%) and frameshift insertion (1/11 - 11.1%). One out of seventeen (5.9%) patients

demonstrated allele deletion but the remaining gene was not mutated. There were two cases of LOH with no mutation in the remaining allele.

Considering *TP53* mutations in OSCC, there is insufficient statistical evidence to assert a significant association between smoking and LOH (Table 1),

Table 01: Association between smoking and TP53 LOH in patients with carcinoma and leukoplakia.

	Loss of Heterozygosity		
	Mutation in both alleles	LHO	P value
Smoking OSCC	0	14	0.471
Non smoking OSCC	0	11	
Smoking OL	3	0	1
Non smoking OL	4	1	

or between smoking and the types of TP53 gene mutations in the analyzed sample (Table 2).

Table 02: Association between smoking and the types of TP53 mutations in patients with carcinoma and leukoplakia.

	Types of mutation			
	Frameshift deletion	Frameshift insertion	Missense	P value

Smoking OSCC	2	0	12	0.136
Non smoking OSCC	2	1	6	
Smoking OL	2	0	1	0.919
Non smoking OL	3	0	2	

PTEN LOH was detected in 13/18 (61.1%) of smokers cases. The most frequent pattern of mutation was missense (8/13 - 72.7%), followed by nonsense mutation (2/13 - 18.2%) and exon deletion (1/13 - 9.1%). Two patients (11.1%) exhibited homozygous mutations, indicated by exon deletion and nonsense mutation. In two patients (2/18 - 11.1%), LOH was identified without mutations in the remaining gene.

PTEN LOH was detected in 6 of the 17 cases from non-smokers (35.3%). The most frequent mutation was missense (4/6 - 66.7%), followed by exon deletion (2/6 - 33.3%).

Considering PTEN LOH in OSCC, there is sufficient statistical evidence to support a significant association between the smoking habit and LOH in the PTEN gene in the analyzed sample (Tables 3),

Table 03: Association between smoking and LOH in PTEN in patients with carcinoma and leukoplakia**.**

	Loss of Heterozygosity		
	Mutation in both alleles	LOH	P value
Smoking OSCC	2	13	0,00686

			5
Non smoking OSCC	0	6	
Smoking OL	4	7	**0,07656**
Non smoking OL	3	3	

however There is not enough statistical evidence to support the existence of a significant association between smoking and the type of PTEN mutation in the analyzed sample (Table 4).

Table 04: Association between smoking and types of PTEN mutations in patients with carcinoma and leukoplakia.

Lesão	Types of mutation			
	Exon deletion	Missense	Nonsense	P value
Smoking OSCC	2	8	3	0,08486
Non smoking OSCC	2	4	0	
Smoking OL	2	0	6	0.032
Non smoking OL	0	0	15	

AKT LOH in smokers was detected in five patients (5/18 - 11.1%) and these cases showed AKT p.E17K. There also was one case (1/18 - 5.6%) of homozygous mutation, showing the activating mutation AKT p.E17K. In three patients (3/18 - 16.7%), we identified LOH but the remaining gene was not mutated.

We identified LOH for *AKT* in 4 of the 17 cases from non-smokers (5.9%). The mutation in the remaining allele was activating mutation AKT p.Q79K. Three (3/17 - 17.7%) of the non-smoker cases demonstrated LOH without mutations in the remaining gene. One case showed homozygous missense mutation (AKT p.E17K).

Considering AKT LOH in OSCC, there is insufficient statistical evidence to support a significant association between smoking and LOH in the AKT gene in the analyzed sample (Table 5),

Table 05: Association between smoking and LOH in AKT in patients with carcinoma and leukoplakia.

	Loss of Heterozygosity		
	Mutation in both alleles	LOH	P value
Smoking OSCC	1	5	1
Non smoking OSCC	1	4	
Smoking OL	1	0	0.6131
Non smoking OL	3	1	

nor between smoking and the type of AKT mutation (Table 6).

Table 06: Association between smoking and the types of AKT mutations in patients with carcinoma and leukoplakia.

	Types of mutation			
	E17K	L52 R	Q79K	P value
Smoking OSCC	3	0	0	0,6026
Non smoking OSCC	1	0	1	
Smoking OL	0	1	0	**0,6131**
Non smoking OL	2	2	0	

Samples from two smokers simultaneously harboured *PTEN*, *TP53* and *AKT* mutation, 8 out of 18 (44.4%) had a *PTEN/TP53* concomitant mutation, one (1/18 - 5.9%) had a *PTEN/AKT* concomitant mutation.

Two non-smoker patients' cases (2/17 - 11.8%) had *TP53/PTEN* co-occurring mutations, one (1/17 - 5.9%) had a *TP53/AKT* co-occurring mutations and one sample (1/17 - 5.9%) had a *PTEN/AKT* concomitant mutation.

Oral Leukoplakia molecular results

Considering smokers patients, LOH at *TP53* was not observed. In 3/17 cases, both alleles were mutated and the most frequent mutation was frameshift deletion (2/3 - 66.7%), followed by missense (1/3 - 33.3%). Meanwhile in non-smokers, one sample (1/21 - 4.8%)

showed LOH for *TP53* (nonsense mutation). Homozygous mutations were detected in four cases (4/21 - 19.1%), and the most frequent pattern of mutation was frameshift deletion (3/4 - 75%), followed by nonsense mutation (1/4 - 25%). There is insufficient statistical evidence to support a significant association between tobacco use and the types of mutations in the TP53 gene in the analyzed sample (table 2) nor between smoking and the LOH (table 1).

7/17 (41.2%) of the smokers samples showed LOH at *PTEN*, all missense mutations. Four cases (4/17 - 23.5%) had homozygous mutation, of which two (2/4 - 50%) showed missense mutation and the other two cases (2/4 - 50%), exon deletion. In non-smokers, LOH at *PTEN* was found in 3 out of 21 cases (14.3%). Missense mutation was detected in all remaining alleles. In addition, three cases (3/21 - 14.3%) demonstrated homozygous missense mutation. There is sufficient statistical evidence to support a significant association between the smoking habit and types of mutations in the PTEN gene in the analyzed sample of oral leukoplakia (table 4), however there is not enough statistical evidence to support the existence of a significant association between smoking and LOH (Table 3).

LOH at *AKT* was not observed in the OL samples from smokers. One out of the 17 smokers (5.9%) showed *PTEN* and *AKT* mutation, the last one being the activating mutation AKT p.L52R. In non-smokers, LOH at AKT was detected in one case (1/21 - 4.8%) and the remaining allele showed the activating mutation AKT p.E17K. In three cases (3/21 - 14.3%), homozygous mutation was detected, two demonstrating activating mutant p.L52R and one demonstrating activating mutant AKT p.E17K. There is insufficient statistical evidence to support a significant association between the tobacco use and type of mutation in the AKT gene in the analyzed sample (Table 6), or between smoking and LOH (Table 5).

Regarding smokers patients, two samples (11.8%) exhibited simultaneous mutations in *PTEN* and *TP53*. In non-smokers, one sample (1/21 - 4.8%) demonstrated co-occurrence of

TP53 and *AKT* mutations, while another sample (1/21 - 4.8%) displayed a double mutation in *PTEN* and *AKT*.

DISCUSSION

OSCC remains a significant health problem [13], leading to a poor survival rate, high morbidity and mortality [14]. Considering that the smoking habit is a well-established risk factor for both OPMD and OSCC [13], in this study we investigated LOH, the frequency and patterns of the mutations in the *TP53*, *PTEN* and *AKT* genes in both TSCC and OL tissues of smokers and non-smokers.

Regarding tobacco-associated TSCC, our results show *TP53* as the most frequently mutated gene (77.8%), consistently with previous reports [15]. In agreement with findings by Nagakagi et al and Hyodo et al [16, 17], this study demonstrates that the *TP53* mutation site is mostly localized in the DNA binding Domain (DBD), and that missense pattern of mutation is predominant (85.7%). Although truncating mutations are more common outside the DBD [16], two patients exhibited missense mutations at codon 45, in the Transactivation Domain 2 (TAD2). In the DBD, the TP53 mutations found on codons 193, 220 and 248 had been formerly demonstrated on OSCC [16].

In non-smokers TSCC patients, *TP53* was also the most recurrently mutated gene, but the frequency of mutation was lower than in tobacco-associated TSCC. All cases exhibited LOH in the DBD region on codons 193, 203, 220 and 248 and missense was the most common mutation. Therefore, LOH for *TP53* was more frequent in smokers and missense mutations were predominant in both smokers and non-smokers.

PTEN was the second most frequently mutated gene in tobacco-associated TSCC, with a frequency of 72.2%. LOH at *PTEN* was detected in 61.1% of cases. Missense mutation was identified as the most frequent pattern of mutation in the remaining allele, differently to the

findings of Kato et al [18], in which nonsense pattern was predominant. In the cases where homozygous mutation was detected, frameshift deletion and missense patterns were identified.

Similarly to tobacco-associated TSCC, *PTEN* was the second most frequently mutated gene in non-smoker patients with TSCC. However, the frequency of mutation was lower compared to smokers. All cases showed LOH and the missense pattern of mutation was predominant in the remaining gene.

AKT1 was found to be the least frequently mutated gene in smoking TSCC patients. Although past studies have pointed to high expression of p-AKT as correlated with a poor prognosis in OSCC, there is insufficient evidence to support *AKT1* gene mutation in OSCC [19]. Deletion not followed by mutation was detected in 16.7% of cases, and LOH occurred in an equal proportion of cases. In all cases of LOH, we detected activating mutant AKT p.E17K, which is a rare event in oral carcinogenesis, though it is the most common hotspot mutation in other malignancies [20]. No patient showed *AKT1* mutation alone, either it was a double (AKT/PTEN) or a triple mutation.

In non-smoker TSCC patients, *AKT1* was the least mutated gene as well, with a lower frequency of mutation compared to smokers. Missense mutation was the only pattern present. Similarly, any case showed *AKT1* mutation alone, only concomitant *AKT/PTEN* and *AKT/P53* mutations. Therefore, LOH was found to be infrequent in both smoking and non-smoking patients, suggesting it is not a highly prevalent event in oral carcinogenesis.

Regarding simultaneous mutations, smoking patients exhibited a greater number of double mutations in *PTEN* and *TP53* when compared to non-smoking patients (8 out of 18 patients and 2 out of 17 patients respectively). Moreover, smoking patients presented two cases with a triple mutation (*TP53*, *PTEN* and *AKT*), while non-smoking patients did not

present any. This discrepancy suggests a possible difference in oral carcinogenesis between smokers and non-smokers.

Concerning tobacco-associated OL, *PTEN* was the most frequently mutated gene (64.7%). Although loss of *PTEN* function in OL has been previously reported [3,21], our findings are in disagreement with the literature on this subject, as *TP53* and *KMT2C* have been described as the most frequently mutated genes in OL [22]. LOH was significantly more frequent than homozygous mutation, and missense pattern of mutation was predominant.

PTEN was the most recurrent mutated gene in OL of non-smokers as well (28.6%). However, the frequency of LOH and homozygous mutation was the same. Missense was the only pattern of mutation identified. Therefore, in non-smoking patients, allelic loss in the *PTEN* gene in both carcinomas and OL occurs less frequently compared to smoker patients. This difference reveals a distinction in oral carcinogenesis between these patient groups. However, a common thread is the predominance of missense mutations.

Considering that *PTEN* LOH in smoking patients occurs both in OL (41.2% of cases) and in TSCC (61.1% of cases), it is clearly an early event in oral carcinogenesis. Additionally, the frequency of the missense characterizes it as the main mutation in the process of acquiring the oral malignant phenotype from leukoplakia.

In this research, *TP53* was the second most recurrently mutated gene in OL of smokers (17.6%) and non-smokers (23.8%). Its frequency of mutation was significantly lower than PTEN in smokers and nearly the same as PTEN in non-smokers. In tobacco-associated OL, homozygous mutations were unanimous, with frameshift deletion being more frequent than missense mutation. Conversely, in non-smokers, homozygous mutations were predominant, with frameshift deletion being the most frequent mutations, followed by nonsense.

In both smokers and non-smokers OL patients, *TP53* LOH was infrequent, with only one case observed in non-smokers and none detected in smokers. Furthermore, only a few cases were mutated in the two groups. Therefore, it can be concluded that LOH and the presence of mutation in the studied samples do not represent an event that occurs early in oral carcinogenesis via OL in both smokers and non-smokers.

AKT1 was the least frequently mutated gene in OL of both smokers (5.9%) and non smokers (19%). In tobacco associated OL, only one homozygous missense mutation was present, which was a concomitant *AKT/PTEN* mutation. However, in non-smokers OL, although *AKT1* mutations were less frequent, their frequency was higher than in smokers, leading to the conclusion that AKT1 may play a stronger role in OL not associated with tobacco. Furthermore, the non-smoking group was the one in which we identified samples where *AKT1* was the only mutated gene, all of them presenting homozygous missense mutations. LOH for *AKT* in OL was not observed in smokers, and only one case was detected in the non-smoker group. Overall, as few mutations for *AKT1* were detected, it is deduced that the main alteration in the PI3K pathway in oral carcinogenesis in the cases studied arises especially from alterations in the *PTEN* gene.

In conclusion, the present study suggests that there are differences between the mutation frequency in the *TP53, PTEN* and *AKT* genes in smoking and non-smoking patients. Changes in the TP53 gene were less frequent in the OL stage than in OSCC, with no statistically significant difference between smoking and non-smoking patients, whereas PTEN gene alterations in both OL and OSCC showed a statistically significant difference between smokers and non-smokers. Furthermore, changes in the AKT gene did not occur with high frequency in either OL or carcinomas, smokers or non-smokers, demonstrating that changes in the important PI3K pathway are primarily due to genetic changes in PTEN rather than in the AKT gene.

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CONFLICT OF INTEREST

The authors state that they have no potential conflict of interest that could bias the results obtained in the current study.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Ethical Committee of the Piracicaba Dental School, University of Campinas, Piracicaba, Brazil (process number 6.010.777).

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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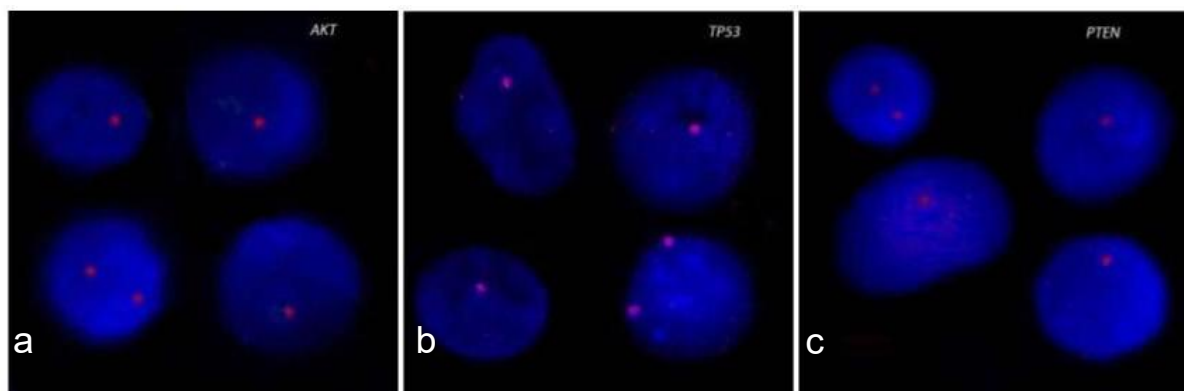
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Figure 1. Fluorescence in situ hybridization (FISH) for *AKT* (a)*, TP53* (b) and *PTEN* (c).