Spermidine Suppresses Oral Carcinogenesis through Autophagy Induction, DNA Damage Repair, and Oxidative Stress Reduction

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Autophagy has been proposed to play a dual role in cancer—as a tumor suppressor in early stages and oncogenic in late stages of tumorigenesis. This study investigated the role of autophagy in oral carcinogenesis using the model of oral squamous cell carcinoma (OSCC) induced by carcinogen 4-nitroquinoline 1-oxide (4NQO), mimicking molecular and histopathologic aspects of human OSCC. The induction of autophagy by spermidine (SPD) treatment reduced the severity of lesions and the incidence of OSCC in mice exposed to 4NQO. On the other hand, autophagy inhibition by chloroquine treatment had no protection. The comet assay indicated that SPD reduced 4NQO-induced DNA damage, likely related to the activation of DNA repair and the decrease of reactive oxygen species. As sphingolipid alterations have been reported in OSCC, sphingolipids in the tongue and plasma of animals were analyzed and plasma C16 ceramide levels were shown to increase proportionally to lesion severity, indicating its potential as a biomarker. Mice exposed to 4NQO plus SPD had lower levels of C16 ceramide than the 4NQO group, which indicated SPD’s ability to prevent the 4NQO-induced carcinogenesis. Together, these data indicate that activation of autophagy has a tumor suppressor role during the early stages of oral carcinogenesis. Because of its ability to induce autophagy accompanied by reduced oxidative stress and DNA damage, SPD may have a protective action against chemically induced oral cancer. (Am J Pathol 2023, 193: 2172–2181; https://doi.org/10.1016/j.ajpath.2023.09.005)

Oral squamous cell carcinoma (OSCC) is highly aggressive and often metastasizes to cervical lymph nodes.1,2 Despite advances in surgical practices, therapies, and increased knowledge about the causes and risk factors associated with OSCC, the 5-year survival rate for advanced OSCC has not improved substantially in recent years. Consequently, new forms of early diagnosis and therapeutic intervention are needed.2,3

Autophagy is a cellular process that permits damaged molecules and organelles to be transported to autophagosomes for degradation and recycling, safeguarding the energetic homeostasis of the cell.4 The role of autophagy in cancer is considered dual, and is the opposite depending on the stage of the carcinogenic process. At early stages of tumorigenesis, autophagy may behave as a tumor suppressor by destroying dysfunctional mitochondria and protein aggregates, thus preventing excessive production of reactive oxygen species (ROS) that can increase the rate of DNA damage and the associated mutations.5 However, at the later stages of tumorigenesis, autophagy can play an oncogenic role because of its capacity to support the survival and growth of tumor cells under stressful conditions, such as hypoxia and attack by the immune system.6,7

Modulation of autophagy in OSCC, either decreasing or enhancing it, has been suggested to inhibit OSCC.8,9

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However, these studies are based on in vitro assays or xenograft models that cannot reproduce tumor microenvironment characteristics. Herein, the objective was to analyze the role of autophagy (inhibition/activation) in oral carcinogenesis using the model of OSCC induced by treatment with 4-nitroquinoline 1-oxide (4NQO), which mimics mutations, development, and metastasis of human OSCC in tongue and allows investigations in an unaltered tumor microenvironment.10,11

Herein, inhibition of autophagy by chloroquine (CQ) did not inhibit oral carcinogenesis. However, activation of autophagy by spermidine (SPD) inhibited or delayed the development of OSCC induced by 4NQO. These data indicate a tumor suppressor role for autophagy in the early stages of oral tumorigenesis.

Materials and Methods

Chemically Induced Tongue Squamous Cell Carcinoma in Mouse Model

The Ethics Committee for Animal Use in Research of the University of São Paulo, Ribeirão Preto (São Paulo, Brazil), approved animal care and experimental procedures. To evaluate the role of autophagy in oral carcinogenesis, the authors used the model of tongue squamous cell carcinoma induced by the carcinogen 4-NQO. Briefly, wild-type C57BL/6 mice, male (n = 40), were distributed into four experimental groups (10 animals per group). The control group (group 1) of animals was maintained only with pure drinking water; group 2 was treated with 4NQO at a concentration of 100 μg/mL in drinking water; group 3 was treated with 4NQO (100 μg/mL) plus an autophagy inducer (3 mmol/L spermidine; Sigma-Aldrich, St. Louis, MO) in drinking water; and group 4 was treated with 4NQO (100 μg/mL) plus an autophagy inhibitor (0.29 mg/mL chloroquine; Sigma-Aldrich) in drinking water. The animals were allowed to drink ad libitum, and drinking water was renewed weekly. Adequate precautions were taken to prevent the compounds from breaking down on exposure to light. After 10 weeks of exposure to the compounds, the animals were maintained for 16 weeks with pure drinking water. At the end of the experiment, mice were euthanized, blood samples were collected, and the tongues were resected and frozen in liquid nitrogen for molecular analysis or collected, and the tongues were resected and frozen in liquid nitrogen for molecular analysis or

Cell Lines and Culture Conditions

The oral keratinocyte spontaneously immortalized cell line (NOK-SI, named NOK) was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL; Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% CO2.

RNA Interference Using shRNA

NOK cells with stable expression of the shRNA against human ATG12 (NM_004707) or nontargeting shRNA were produced using a pLKO.1puro lentiviral plasmid (Sigma-Aldrich). The target sequence for shRNA against human ATG12 was 5’-TGTGGGCTCTCACTCTCAAA-3’. The sequence of the nontargeting shRNA (SHC002) was 5’-CAACAAAGATGAGAGCACCAC-3’. shRNA lentivirus particles and cells were prepared as previously described.12

Immunoblotting

The total proteins (20 to 40 μg) were separated on SDS-PAGE gel and transferred onto polyvinylidene difluoride membranes. Antibodies used for immunoblotting are listed in Table 2. Immunocomplexes were visualized using electrochemiluminescence reagents (GE Health Care, Freiburg, Germany), and images were acquired using the ChemiDoc MP imaging system (BioRad, Hercules, CA).

Comet Assay

The alkaline comet assay was used to determine the antigenotoxic potential of the SPD in the oral keratinocyte cell line. Briefly, 1 × 10^5 cells per well were seeded in 6-well plates. Next, cells were treated with spermidine (5 μmol/L) for 16 hours and then with spermidine (5 μmol/L) plus 0.2 μg/mL of 4NQO for 24 hours. The treated cells were fixed on slides and submitted to lysis, alkali unwinding, and alkali electrophoresis, followed by neutralization and fixation.13 The slides were stained with 5 μg/mL DAPI per slide. On fluorescence microscopy, 50 cells per slide were randomly imaged and analyzed using the OpenComet software tool version 1.3 (https://cometbio.org).

ROS Detection

The CM-H2DCFDA probe (Thermo Fisher Scientific, Waltham, MA) was used to measure ROS levels in cells, as...
previously reported.\textsuperscript{14} Cells were seeded in a 96-well plate. After 24 hours, cells were incubated with 5 $\mu$mol/L CM-H2DCFDA for 30 minutes, 1 hour, and 2 hours at 37°C in the dark. Cells were washed with phosphate-buffered saline and analyzed using a Synergy two-plate reader (BioTek, Winooski, VT). The experiments were performed in triplicate.

Liquid Chromatographic—Triple Quadrupole Tandem Mass Spectrometry (LC-QqQ-MS/MS) Quantitative Analysis of Sphingolipids

Biological samples from mice exposed to 4-nitroquinoline-1-oxide—induced oral cancer and other treated groups were extracted using a combination of two previously reported protocols.\textsuperscript{15,16} The quantification in tissue samples was normalized by weight. Sequentially, the tongue tumor tissues (20 $\pm$ 5 mg) were crushed in methanol (1 mL) and transferred to a 5-mL glass tube (tube number 0), as well as blood plasma samples (100 $\mu$L), and the FTY720 internal standard was added (3.5 ng). The samples were sonicated for 1 minute (tube number 0) using 1.5 mL of methanol:chloroform (2:1; v/v), centrifuged at 3000 $g$ for 5 minutes. The supernatant was transferred to the new glass tube (tube number 1). Finally, chloroform (1 mL) was added to tube number 0 and centrifuged under the same conditions, and the lower organic phase (1 mL) was transferred to tube number 1. The total volume of tube number 1 was dried and reconstituted in chromatographic solution (phase A:phase B; 2:8; v/v) and centrifuged at 2218 $g$ for 5 minutes. The supernatant was transferred to a glass vial for analysis. The analysis of sphingolipids was performed on LC-QqQ-MS/MS (Shimadzu, Kyoto, Japan) coupled to an API3200 triple-quadrupole mass spectrometer (AB Sciex, Toronto, ON, Canada), with chromatographic and mass spectrometry conditions previously reported.\textsuperscript{15} The Sphingolipid Mix II Standard Mixture (25 $\mu$mol/L; number LM6005; AVANTI Lipids, Alabaster, AL) added with the internal standard (FTY720; Sigma-Aldrich) was used to obtain a calibration curve (20 to 2000 pmol/mL).\textsuperscript{16}

### Table 1

<table>
<thead>
<tr>
<th>Score</th>
<th>Characteristics</th>
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<tbody>
<tr>
<td>0</td>
<td>Dysplasia absent</td>
</tr>
<tr>
<td>1</td>
<td>Mild dysplasia (alterations limited to one-third of the basal epithelium)</td>
</tr>
<tr>
<td>2</td>
<td>Moderate dysplasia (alterations representing two-thirds of the epithelium)</td>
</tr>
<tr>
<td>3</td>
<td>Severe dysplasia (alterations representing more than two-thirds of the epithelium)</td>
</tr>
<tr>
<td>4</td>
<td>Carcinoma in situ (changes in the entire epithelial thickness, but without disruption of the basement membrane)</td>
</tr>
<tr>
<td>5</td>
<td>Invasive carcinoma (carcinomatous islands within connective tissue)</td>
</tr>
</tbody>
</table>

### Table 2

<table>
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<tr>
<th>Antibody (catalog no.)</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRCA1 (9025)</td>
<td>Cell Signaling Technology (Danvers, MA)</td>
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<tr>
<td>PARP (9532)</td>
<td>Cell Signaling Technology</td>
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<tr>
<td>MLH1 (3515)</td>
<td>Cell Signaling Technology</td>
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<td>PCNA (2586)</td>
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<tr>
<td>GAPDH (2118)</td>
<td>Cell Signaling Technology</td>
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<tr>
<td>Acetyl-p53 (Lys382) (2525)</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>LC3 (3868)</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td>SET (SAB4200479)</td>
<td>Sigma-Aldrich (St. Louis, MO)</td>
</tr>
<tr>
<td>$\beta$-Actin (sc-47778)</td>
<td>Santa Cruz Biotechnology (Dallas, TX)</td>
</tr>
<tr>
<td>SQSTM1 (D-3) (p62 (sc-28359)</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>p-H2A.X (sc-517348)</td>
<td>Santa Cruz Biotechnology</td>
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</table>

BRCA1, breast cancer type 1 susceptibility protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LC3, microtubule-associated protein 1 light chain 3; MLH1, Mut. homolog 1; PARP, poly (ADP-ribose) polymerase; PCNA, proliferating cell nuclear antigen; p-H2A.X, phospho-H2A.X variant histone; SET, SET nuclear proto-oncogene; SQSTM1/p62, sequestosome 1.

### Statistical Analysis

Comparisons between the two groups were performed with the unpaired $t$-test. Comparisons between three or more groups were performed using the Kruskal-Wallis test. In all statistical analyses, $P < 0.05$ was considered statistically significant. Heat map and box-dot plots were drawn in RStudio (Posit, Boston, MA) using the Pheatmap package version 1.0.12 (https://cran.r-project.org/web/packages/pheatmap/index.html) and the Ggplot2 package version 3.4.1 (https://cran.r-project.org/web/packages/ggplot2/index.html), respectively.

### Results

Enhanced Autophagy Induced by Spermidine Reduces Oral Carcinoma Development in a 4NQO Mice Model

To examine the role of autophagy in OSCC development, C57/BL6 mice were exposed to 4NQO diluted in drinking water and compared with the groups exposed to 4NQO plus
autophagy inhibitor (4NQO + CQ) or 4NQO plus autophagy inducer (4NQO + SPD) diluted in drinking water for 10 weeks. All animal groups were maintained at normal drinking water for additional 16 weeks. As shown in Figure 1A, there was a reduction in the final weight of mice exposed to 4NQO, which may be related to lower food consumption due to tongue lesions. Interestingly, the weight of mice treated with 4NQO + SPD was similar to that of the control group. However, although the group with 4NQO + CQ had severe lesions, they did not experience any significant weight loss. This could be because CQ has a possible impact on fat metabolism, as these animals had an accumulation of abdominal fat (Supplemental Figure S1). Moreover, although the activation of autophagy by SPD reduced the lesion areas on the tongue surface, inhibition of autophagy by CQ did not show a reduction compared with...
that in the 4NQO group (Figure 1B). Microscopically, severe epithelial dysplasia and OSCC appeared in the tongue of mice treated with the 4NQO or 4NQO + CQ. In contrast, the 4NQO + SPD group had less severe tongue lesions. Most lesions exhibited mild-to-moderate epithelial dysplasia, as indicated in Figure 1, C and D, and Supplemental Figures S2 through S4. On the basis of these findings, it appears that increasing autophagy using SPD can slow down or decrease the progression of OSCC. The proliferative (SET and proliferating cell nuclear antigen) and autophagy (p62) markers were analyzed in the tongues by using immunohistochemistry for SET (Figure 1E) and Western blot analysis for proliferating cell nuclear antigen and p62 proteins (Figure 1F). Tissue slices and proteins of tongues from mice treated with SPD had reduced SET and proliferating cell nuclear antigen protein levels compared with those in the 4NQO group. The levels of p62 protein varied widely among the control and 4NQO groups. However, all three animals in the CQ group had the lowest levels, whereas all three animals in the SPD group had high p62 levels (Figure 1F). These data suggest a reduction of cell proliferation in tongue tissue in mice treated with 4NQO + SPD when compared with severe dysplastic and OSCC tissues in mice exposed to 4NQO. Moreover, autophagy marker (p62) was consistently suppressed in the 4NQO + CQ group, whereas it was present in SPD group.

Spermidine Prevents Oxidative Stress and DNA Damage

4NQO is a carcinogen that can cause DNA damage by forming DNA adducts and by generating ROS that cause oxidative DNA damage. Therefore, whether the antitumor potential of spermidine was linked to the ability to reduce DNA damage was investigated next. The comet assay indicated that SPD could reduce DNA damage caused by 4NQO in oral nontumoral keratinocytes (NOK) (Figure 2, A and B). Next, the mechanisms involved in this effect of SPD were investigated, as shown in Figure 2C. SPD by itself (under conditions without DNA damage) induced the expression of proteins involved in DNA repair pathways, such as poly (ADP-ribose) polymerase (PARP), breast cancer type 1 susceptibility protein (BRCA1), sequestosome 1 (p62), microtubule-associated protein 1 light chain 3 beta (LC3B), and acetylated p53. In the presence of 4NQO, there was a decrease in the expression of MutL homolog 1 (MLH1) and PARP, and an increase in cleaved SET, LC3B, and acetylated p53. In the 4NQO group, a NOK cell line with autophagy related 12 (ATG12) protein silencing was generated. As shown in Figure 2E, the increase in acetylated p53 and phospho-H2A.X variant histone (H2AX) was correlated with autophagy after 4NQO-induced damage. Therefore, the more autophagy, the greater the damage. This indicates that the response to 4NQO-induced damage depends on autophagy, and SPD-activated autophagy protects against 4NQO-induced carcinogenesis.

Spermidine Prevents Sphingolipid Dysregulation in Mice Oral Carcinogenesis

Sphingolipid metabolism pathways are dysregulated in several cancer types, including oral carcinoma. Additionally, sphingolipids may participate in the DNA damage response and autophagic processes. Therefore, sphingolipid profile was evaluated in the tongue and plasma from four mice groups (control, 4NQO, 4NQO + CQ, and 4NQO + SPD). As shown in the heat map in Figure 3A, on the basis of the profile of five sphingolipids in the tongue, animals treated with 4NQO and 4NQO + CQ tended to cluster together, whereas animals treated with 4NQO + SPD were closer to the control group. Among the altered sphingolipids, ceramide C16 was increased in both the tongue and the plasma of mice treated with 4NQO compared with the control group (Figure 3, B and C). The level of C16 (tissue and plasma) was higher in the group treated with 4NQO + SPD compared with the control group, but lower compared with the 4NQO group, which may be related to the fact that SPD reduced the lesions caused by 4NQO. A significant increase in C16 glucosylceramide was observed in the plasma of 4NQO-treated group (Figure 3D). This glycosylated form of ceramide is present in plasma at much higher levels than unmodified ceramide. Analysis of the C16 ceramide profile according to the degree of injury showed its level to be proportional to the degree of damage, regardless of the group, suggesting that the increase in ceramide was directly related to the tissue lesions and cell transformation (Figure 3E).

Discussion

This is the first study to elucidate the role of autophagy as a tumor suppressor during the early steps of oral carcinogenesis using an in vivo model. Autophagy ensures cell homeostasis by purifying and recycling possibly damaging cellular components related to its protective function against
diseases, including cancer. The currently most accepted hypothesis is that autophagy has a suppressive effect on the initial stages of the carcinogenic process because of its ability to eliminate damaged cells that could later undergo a malignant transformation process. To test the theory, the murine model of oral carcinogenesis induced by 4NQO was selected. This model closely mimics the morphologic, pathologic, and molecular aspects of human OSCC. However, it has some limitations, including high heterogeneity in induced malignant lesions and an inability to investigate the role of specific genes in the development of oral carcinoma. In addition, identifying lesions within the oral cavity of the mice can be challenging. As a result, the evaluation of tumor development and treatment effects is typically done through histopathology after removing the tongue from the animals. Herein, autophagy inhibition was achieved with chloroquine, which prevents autophagosome-lysosome fusion and has been tested in cancer clinical trials as an adjuvant compound associated with chemotherapy. Autophagy was induced by treatment with spermidine, a naturally occurring polyamine, which can extend the lifespan of yeast, flies and worms, and human immune cells. Oral spermidine supplementation can induce autophagy in different tissues, at least partly related to its anti-disease and anti-aging effects. Mice treated with chloroquine and exposed to 4NQO had an incidence of carcinoma on the tongue, similar
to that in mice exposed to 4NQO alone. On the other hand, animals treated with spermidine + 4NQO had a lower incidence of tongue carcinoma than the 4NQO group. In the SPD + 4NQO group, most tongue lesions were stopped at the epithelial-moderate dysplastic stage, indicating that spermidine-induced autophagy had an inhibitory effect on the development of oral cancer (Figure 4).

Supplementation with SPD or polyamine reduced the incidence of hepatocellular carcinomas in mice treated with the hepatocarcinogen diethylnitrosamine and the incidence of colon cancer in BALB/c mice treated with the carcinogen 1,2-dimethylhydrazine.

These data elucidate the tumor suppressor role of autophagy in the oral carcinogenic process. A study of molecular mechanisms related to the antitumor effect of SPD indicated that SPD reduced DNA damage caused by 4NQO in oral keratinocytes. Furthermore, SPD activated important proteins in DNA repair, such as BRCA1 and PARP, which are essential in recruiting effector repair proteins to DNA damage sites.

SPD also reverses the negative effect of 4NQO on the expression of repair proteins, such as PARP and MutL homolog 1, an essential protein of the mismatch repair pathway. In addition to repairing mispaired bases during DNA replication, the mismatch repair pathway can also repair oxidative DNA damage. Herein, SPD increased p62, a cargo receptor for the degradation of ubiquitinated protein aggregates by autophagy, confirming its activation and action on other processes, such as improving the efficiency excision repair pathway through recruitment of apurinic/apyrimidinic endodeoxyribonuclease 1 (APE1) to sites of DNA damage. APE1 plays a crucial role in removing oxidatively damaged bases. Thus, it may have a role in processing oxidative DNA damage induced by

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**Figure 3** Oral carcinogenesis in 4-nitroquinoline 1-oxide (4NQO) mice model reveals a dysregulation in metabolism of sphingolipids mainly in ceramides and sphingosine-1-phosphate. A: Heat map showing profiles of five sphingolipids in tongue from mice exposed to 4NQO, 4NQO + spermidine (SPD), 4NQO + chloroquine (CQ), and control group. B: Level of C16 ceramide in the tongue tissue. C and D: Plasma level of C16:0 ceramide (C) and C16:0 glucosylceramide (D). E: Plasma levels of C16 ceramide based on the severity of tongue lesions (grade 0 to V).
4NQO. However, SPD reduced both SET and APE1 proteins in tongue tissues. SET is a deregulated protein in oral cancer, which can inhibit DNA repair in double-stranded break regions through increasing chromatin compaction that prevents the recruitment of DNA repair factors, such as BRCA1 and RAD51 recombinase (RAD51). Therefore, in addition to its ability to induce autophagy, SPD appears to have the potential to reduce DNA damage by enhancing DNA repair mechanisms. In bacteria, SPD reduces the level of mutations induced by 4NQO.

Oxidative DNA damage appears to be an early event during oral carcinogenesis induced by 4NQO in oral keratinocytes. Herein, SPD reduced the level of reactive oxygen species generated by 4NQO and slightly diminished baseline ROS levels of oral keratinocytes. Consequently, lower SET and APE1 level compared with 4NQO was expected. Therefore, the antioxidant properties of SPD may play a relevant role in protecting against carcinogenesis induced by chemical insults, which is in line with the potential of SPD to reduce damage caused by oxidative stress in different disease models.

Sphingolipids function in the autophagy and DNA damage response, and their levels are altered by treatment with autophagy modulators, such as hydroxychloroquine. Interestingly, the current study identified alterations in sphingolipids profiles, mainly an increase of C16 ceramide in tongue tissue and plasma, and C16 glucosylceramide in plasma, of animals exposed to 4NQO compared with the control group. In contrast, both C16 ceramides were reduced in the group treated with spermidine + 4NQO. Notably, the level of C16 ceramide increased with the severity of the lesion, suggesting that the increase in ceramide may be directly related to the progression of oral carcinogenesis. Increased C16 ceramide is observed in the plasma of patients with hepatocellular carcinoma. Increased C16 ceramide through overexpression of ceramide synthase 6 increases head and neck squamous carcinoma growth in a tumor xenograft model, corroborating our data.

Besides, as ceramide levels may increase in response to DNA damage and trigger apoptosis of cells with damaged DNA, the increase in C16 ceramide levels may be due to the accumulation of DNA lesions after treatment with 4NQO. However, the accumulation of ceramide may be inefficient in triggering apoptosis due to the increase in C16 glucosylceramide because glycosylation can inhibit the pro-apoptotic activity of ceramide.

In conclusion, these data indicate that autophagy has a tumor suppressor effect during the early stages of oral tumorigenesis, mainly by decreasing oxidative stress and DNA damage and keeping cellular homeostasis, as evidenced by sphingolipid metabolism. It highlights the chemopreventive actions of spermidine against oral carcinogenesis.

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**Disclosure Statement**

None declared.

**Supplemental Data**

Supplemental material for this article can be found at http://doi.org/10.1016/j.ajpath.2023.09.005.

**References**


