Clear cell carcinoma of salivary gland. Clinicpathologic study of 11 cases

Glauber Bareia Liberato Rocha (glauber.rocha@hc.fm.usp.br)
University of São Paulo – USP
Wladimir Gushiken de Campos
University of São Paulo – USP
Fabio Daumas Nunes[3]
University of São Paulo – USP
Juvani Lago Saturno
University of São Paulo – USP
Celso Augusto Lemos
University of São Paulo – USP
Suzana Cantanhede Orsini Machado de Sousa
University of São Paulo – USP

Research Article

Keywords: Carcinoma, Salivary glands, Adenocarcinoma, clear cell, Polymerase chain reaction, EWSR1 protein, human, Pathology, oral

Posted Date: August 5th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1894948/v1

Abstract

Introduction. Clear cell carcinoma of salivary gland is a rare malignant neoplasm that accounts for less than 1% of salivary gland carcinomas. This term is common in many carcinomas with clear cells; however, it is more specific to hyalinizing clear cell carcinoma. Objective. This research aimed to evaluate histopathological, immunohistochemical, biomolecular, and clinical aspects of clear cell carcinomas of salivary gland cases diagnosed between 1997 and 2018.

Materials and Methods. Histological sections, immunohistochemical reactions for cytokeratins 7 and 14 (CK7 and CK14), p63 protein, S-100 protein, and smooth muscle actin (SMA) were assessed, and real-time molecular polymerase chain reaction (RT-PCR) testing was performed to investigate the expression of the EWSR1-ATF1 fusion gene.

Results. Eleven lesions with histological and immunohistochemical characteristics of clear cell carcinoma of salivary glands were found, 81.81% occurred in women, 77.77% in white people and the mean age was 56.42 years. The most affected regions were palate and buccal mucosa (36.36% of cases in each of these regions). Histologically, nine cases had hyalinization; neural invasion and necrosis were present in 6 and 2 of the cases, respectively. All cases were positive for periodic acid-Schiff (PAS) and expressed p63 protein and cytokeratins 7 and 14. In the PCR molecular test, 6 lesions showed EWSR1-ATF1 fusion gene.

Conclusion. Clear cell carcinoma of salivary glands is more prevalent in women aged between 50 and 60 years. Histology associated with immunohistochemistry often closes the diagnosis. Molecular tests should be used as an aid in diagnosis.

Introduction

In terms of salivary gland neoplasms, the most common benign one is pleomorphic adenoma, while the most frequent malignant neoplasm is mucoepidermoid carcinoma. In the 2017 World Health Organization (WHO) classification, 11 types of benign neoplasms and 20 types of malignant salivary gland neoplasms were reported, with molecular tests playing an important role in defining the diagnosis of some of these neoplasms [1–3].

Clear cell carcinoma of salivary gland is a rare lesion and accounts for less than 1% of salivary gland carcinomas. Histologically, its appearance is similar to several neoplasms that present clear cells, such as clear cell oncocytoma, mucoepidermoid carcinoma, epithelial-myoepithelial carcinoma, clear cell myoepithelial carcinoma, myoepithelial carcinoma, acinar cell carcinoma, metastasis of clear cell renal carcinoma and clear cell melanoma. Thus, the differential diagnosis based only on morphological characteristics is often difficult, requiring the use of complementary methods such as immunohistochemistry or molecular biology. The latter method is nowadays employed to better elucidate both the diagnosis and the biological behavior of neoplasms [1, 3–6].

The first study describing clear cell carcinoma of the salivary glands was authored by Chen [1] in 1983. The study by Milchgrub et al. [4] analyzed 11 cases with very similar characteristics, between 1970 and 1992. These authors were the first ones to name these lesions hyalinizing clear cell carcinoma of the salivary gland. Clinically, they were described as painless slow-growing nodules, mostly from minor salivary glands of the oral cavity (82%), with a predilection for women and a mean age of 55 years. Metastasis was described in only two cases, thus confirming a non-aggressive neoplasm with a low degree of malignancy. The histological characteristics depicted a well-defined lesion with clear cells of polygonal shape, mostly arranged in islands, cords or nests in a hyaline stroma. Some eosinophilic cells were observed alongside the clear cells. The latter were PAS-positive diastasis-sensitive cells, and mucin was rare to find. Mitosis was observed only in cases with metastases, and neural infiltration was noted, but no vascular infiltration was found. Immunohistochemistry showed expression for epithelial membrane antigen (EMA), cytokeratin AE3 and AE1 and negative staining for protein S100 and smooth muscle actin (SMA), thus characterizing it as an epithelial neoplasm, but not myoepithelial [4, 7–12]. Even after that, the lesion was not accepted as a distinct neoplasm by the WHO and was only recognized as clear cell carcinoma of the salivary glands in the latest classification of 2017 [13].

The recognition of this lesion occurred due to the findings of Antonescu et al. [14]; they studied 23 cases of clear cell carcinoma of the salivary glands, histologically observing the presence of neural invasion in 11, no vascular invasion or necrosis, all positive for p63 and cytokeratins, and only one case positive for S-100. These authors observed the presence of EWSR1 gene with its various fusions in clear cell carcinoma of glandular origin and the presence of EWSR1-ATF1 fusion gene in 82% of cases. This gene is not observed in any other salivary gland lesion, which made the diagnosis more precise. In the WHO classification, it is described that the presence of this gene characterizes the diagnosis of this lesion. Another lesion that presents this fusion gene is clear cell odontogenic carcinoma. This lesion has the same histological and immunohistochemical characteristics as clear cell carcinoma of salivary gland, so clinical aspects are very important for this diagnosis once the first lesion is intraosseous and the second only occurs in the mucosa [8, 11, 14–20].

Yang et al. carried out a literature review and found 141 cases of clear cell carcinomas. By searching his service from 2002 to 2012, they found 14 cases, totaling 155 cases. The mean age of patients was 57 years and 63.2% were women, with the tongue being the most frequent site, followed by the palate. The authors observed the rearrangement of the EWSR1 gene with FISH in 12 of 14 cases. Histology showed perineural invasion in 42 of 96 cases (43.8%), angiolymphatic invasion in nine of 71 patients (12.7%), necrosis in 7 of 51 patients and mitosis in 19 of 93 patients. In the follow-up of these patients, lymphadenopathy was rare (only 17.3% of patients), and distant metastasis was reported in only two cases. Of 130 patients who had postoperative reports, 23 (17.7%) had local recurrence, while five out of 128 patients had regional metastases and four out of 114, distant metastases, affecting the lungs. In 105 patients with long-term follow-up, it was observed that 86.7% were free from the lesion, 3.8% died from the disease and 1.9% died from postoperative complications, which depicts a good prognosis for the disease.[21]

In light of the above and with the intention of assisting in the diagnostic parameters of clear cell carcinoma of salivary glands, we proposed this retrospective study on cases diagnosed at the Oral and Maxillofacial Pathology service of the University of São Paulo School of Dentistry.

Material And Methods

All cases diagnosed as clear cell carcinoma not otherwise specified, originating from the salivary gland, were retrieved from the archives of the Oral and Maxillofacial Pathology Service of the University of São Paulo School of Dentistry. Samples from 1997 to 2018 were evaluated. For comparison purposes, some cases with a diagnosis of salivary gland carcinoma with the presence of clear cells, but without a diagnosis of clear cell carcinoma were also included. All clinical data of the patients (age, race, sex) were collected from the request forms, as well as lesion information (location, evolution time, and clinical aspects). All cases were re-evaluated with hematoxylin-eosin (HE) stained slides, and new sections were made when necessary. Immunohistochemical reactions were performed and staining with PAS with and without diastase. Cases with available material were submitted to real-time polymerase chain reaction (PCR) test. The research project was submitted and approved by a local Research Ethics Committee (n. 2.375.405).

Immunohistochemistry

To define the immunohistochemical profile, reactions were performed with the following antibodies, or existing reactions were re-evaluated: S-100 (DAKO, Saint Clair, CA, USA, clone Z311), SMA (DAKO, Saint Clair, CA, USA, brand, M0851 antibody) and p63 (DAKO, Saint Clair, CA, USA, clone, M7247), cytokeratin 7 (CK7) (Cell Marque, Rocklin, CA, USA, clone M7018) and cytokeratin 14 (CK14) (BioGenex, Freemont, CA, USA, clone MU146-UC).

Immunohistochemical reactions were performed as follows: 3µm sections were obtained with a microtome and placed on silane-coated glass slides, then deparaffinized with xylol heated to 37°C, followed by gradual hydration with up to 80% alcohol. After this step, the glass slides were placed in a suitable buffer (citric acid with pH 6.0 for CK7, CK14 and SMA, EDTA for p63, S-100 does not need treatment) for 30 minutes at 37°C for antigen recovery. Then the slides were cooled in running water, washed with distilled water and placed in a hydrogen peroxide solution with a 1 to 1 dilution with distilled water in order to remove endogenous tissue peroxidase. Next, the slides were washed with TRIS solution and placed in the Dako autostainer machine (Dako Colorado, Inc. CA, USA) for automatic processing of the antibody reaction with their respective dilutions (S-100 1:700, SMA 1:200, CK7 1:500, CK14 1:500, and p63 1:50), then submitted to an incubation period of 30 minutes with the Envision system (EnVision® Dual Link System-HRP, DAKO, Carpinteria, CA, USA) as the second antibody. The chromogen selected for revealing the reactions was diaminobenzidine (DAB, 3,3 - diaminobenzidine), incubated for 10 minutes. After washing, the sections were counterstained with Mayer's hematoxylin, dehydrated in an ascending chain of ethanol, diaphanized in xylol and protected by glass coverslips mounted in an automated system (Tissue-Tek® Film® Coverslipper, Sakura Finetech Inc, Torrance, CA, USA). Positive controls

(normal glandular tissue or internal control) and negative controls (omission of the primary antibody) were included in all reactions.

After the immunohistochemistry and PAS staining reactions, the slides were observed under a microscope by an experienced pathologist. By assessing the distribution of the staining, they were classified as positive or not.

Real-time PCR

Identification of the EWSR1-ATF1 fusion gene characteristic of this neoplasm was performed by real-time PCR.

To perform RNA isolation, the material for this test was extracted from the blocks embedded in paraffin as follows: With a microtome, ten 4 µm cuts were made and placed in a 1.5 ml tube. Deparaffinization was performed by placing 1 ml of xylene in the tube with the sample, vortexing was performed and the xylene was maintained for ten minutes. Then, the tubes were centrifuged at 9300rcf for 3 minutes, and the xylene was carefully removed with the aid of a pipette. This process was repeated twice, ensuring the removal of all the paraffin. Then, 1 ml of ethyl alcohol was placed to remove all the solvent, vortexed and kept for ten minutes. Afterwards, the tubes were centrifuged at 9300rcf for 3 minutes and the ethyl alcohol was carefully removed with the aid of a pipette. The material was dried by incubating at 37°C for 5 minutes with in open tubes.

The MagMAX FFPE DNA/RNA Ultra Kit (A31881 Applied Biosystems) was used to extract the RNA from the deparaffinized material, following all the steps recommended by the manufacturer. This system uses microparticles (beads) that bind to genetic material and are separated from the supernatant using magnets; a magnetic shelf was used for this purpose.

210µl of protease solution were added to 1.5 ml tubes with deparaffinized material, in order to cover the entire tube content for protease digestion. The tubes were incubated for one hour at 55°C and another hour at 90°C. After incubation, the contents were centrifuged at 9300rcf for 3 minutes and the supernatant was carefully removed with the aid of a pipette. 20µl of nucleotide acid binding beads were added to aid RNA separation, then 900µl of RNA binding buffer solution were added and mixed with the aid of a shaker for 5 minutes. After that, the tubes were placed on the magnetic shelf for 2 minutes to stabilize the molecules bound to the beads and the supernatant was carefully removed with the aid of a pipette. 500µl of washing buffer were then added and mixed with the aid of a stirrer for 1 minute, followed by placing the tubes on the magnetic shelf for 2 minutes to stabilize the molecules bound to the beads, and the supernatant was discarded. 500µl of washing solution #2 was then added to the tubes and mixed with the aid of a shaker for 1 minute. Again, the tubes were placed in the magnetic shelf for 2 minutes to stabilize the molecules bound to the beads and the supernatant discarded, the tubes were then kept open for 5 minutes for drying. The next step was to add 100µl of DNase solution to the tubes and mix with the aid of a stirrer for 20 minutes at a temperature of 37°C for a better reaction result. Then, 450µl of RNA rebinding buffer was added and mixed with the aid of a shaker for 5 minutes. Posteriorly,

the tubes were placed in the magnetic rack for 5 minutes to stabilize the molecules bound to the beads and the supernatant was discarded. Finally, 50µl of the elution solution was added and mixed with the aid of a shaker for 5 minutes. This last solution dettached the RNA from the magnetic molecules, so when we put the tubes on the magnetic shelf, the RNA was left in the supernatant. The isolated RNA was carefully removed with the aid of a pipette and placed in another 0.5 ml tube. This RNA was stored in a freezer at a temperature of -80°C.

For cDNA synthesis, 10µl of RNA and High Capacity cDNA Reverse Transcription Kit (Thermo Fisher), were used for the reverse transcription reaction, following the manufacturer's instructions. Firstly, the master mix was prepared, to a final volume of 20µl cDNA, by mixing 2µl RT buffer, 0.8µl dNTP mix, 2µl RT randon primers, 1µl multscribe reverse transcriptase and 4.2µl DNA-free water, giving a total of 10µl. These 10µl of master mix were mixed with 10µl of RNA from each sample and placed in a thermocycler (Thermocycler Matercycler Gradient, Eppendorf AG, Hamburg, Germany) programmed for four cycles (10 minutes at 25°C, followed by 120 minutes at 37° C, 5 minutes at 85°C and a cooling step at 4°C). The samples were stored in a freezer at -20°C until use.

Real-time PCR with the obtained cDNA was performed using the thermocycler 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Sybr Green PCR Master Mix (Applied Biosystems) was used as fluorophore, and for amplification of the EWSR1-ATF1 fusion gene, we used the primers 5'- CAA GGA TTA AAT GAC AGT GTG ACT C -3' (forward) and 5'- CTT TCT GTG AGG AGC CTA TG -3' (reverse). The sequences were synthesized by Integrated DNA Technologies according to Nakano et al. study.[22]

25 µl of Master Mix, 0.5 µl of forward primer, 0.5 µl of reverse primer, 5 µl of sample and 19 µl of DNA-free water were mixed in each 500 µl tube. Reactions were performed in duplicate, and a tube with all reagents except the sample cDNA was used as a negative control. The programmed cycling process was the initial denaturation of 95°C for 10 minutes, followed by 40 cycles of amplification at 95°C for 15 seconds for denaturation, and 60°C for 1 minute for replication. At the same time as the reaction took place, a graph with the melting curve was constructed by the machine. This graph demonstrated whether DNA dissociations in cycles occured normally or if there was any interference such as the formation of dimers. Another plot graph was also formed depicting the increase in the amount of amplification of the tested gene. In this graph, it was possible to observe which samples resulted in amplification and no amplification, then reaching a threshold above which the amplification would be considered a true amplification.

Results

In the period ranging from 1997 to 2018, 68,760 cases were analyzed at the Oral and Maxillofacial Pathology Service of the University of São Paulo, School of Dentistry. Out of these, 797 were salivary gland neoplasms (the most common neoplasm was pleomorphic adenoma with 293 cases, followed by mucoepidermoid carcinoma with 120 cases). For this study, 16 cases were selected, according to the following diagnoses: four hyalinizing clear cell carcinomas, seven clear cell carcinomas, two unspecified

adenocarcinomas, one glycogen-rich clear cell carcinoma, one carcinoma suggestive of mucoepidermoid and one case with diagnosis description of a fragment of mucosa with clear cells suggesting glandular neoplasia. Two cases had the slides, but not the blocks of the original lesion. In these cases, it was not possible to carry out the PCR reactions. However, they were included in the epidemiological data because they had slides in HE and immunohistochemical reactions, one with a diagnosis of clear cell carcinoma and one diagnosed as hyalinizing clear cell carcinoma. The remaining 14 cases had both blocks and slides, making it possible to perform immunohistochemical reactions a second time, if necessary, and PCR analysis.

Of the 16 cases analyzed, 13 were positive for CK7, six for CK14, 10 for p63, two for S100 and one was positive for SMA. PAS revealed 14 positive cases, not resistant to diastasis (Table 1). Thus, 11 cases with histological and immunohistochemical characteristics compatible with clear cell carcinoma of the salivary glands (Figs. 1,2,3,4,5,6,7) were selected and, among these, four had been diagnosed as hyalinizing clear cell carcinoma, five were classified as clear cell carcinoma, one was a mucoepidermoid carcinoma and one was as a mucosal fragment containing clear cells suggesting salivary gland neoplasia. One case diagnosed as clear cell carcinoma was not confirmed by immunohistochemistry and was removed from the samples, and the other two cases diagnosed as clear cell carcinomas were metastases from renal carcinoma. (Table 1)

While analyzing the eleven cases diagnosed as clear cell carcinoma of the salivary glands, it was observed that 81.81% (9 cases) occurred in women, 77.77% (7 out of 9 cases) were white, and the mean age was 56.42 (ranging from 34 to 75) years. The most affected region was the palate together with the buccal mucosa with 36.36% (4 cases each) of the cases. Histologically, 81.81% of the cases presented septa with hyalinization, ranging from large and wide to narrow with some cells. (Fig. 1) All cases had polygonal clear cells arranged in cords, nests, or islands, and sometimes cells with eosinophilic cytoplasm were also observed, varying in their quantity. Neural invasion was observed in six cases (54.54%) (Fig. 6), necrosis was noted in two of the cases (18.18%) (Fig. 7) and lymphovascular invasion was not observed. Real-time PCR was performed in all 14 lesions (in which paraffin blocks were available); among these, 9 were classified as salivary gland clear cell carcinoma. In PCR, 6 of the 9 (66.66%) lesions classified as clear cell carcinoma were positive for EWSR1-ATF1 gene, while the other lesions that had not been classified as clear cell carcinoma were negative for EWSR1-ATF1 gene. The melting curve depicted the normality of the reaction, and the plot graph presented the amplification of the samples (Fig. 8).

Discussion

Most salivary gland neoplasms have a predilection for the female gender, so does clear cell carcinoma. The first study that described this lesion also noticed this propensity for females. Another interesting fact is the mean age occurring between the fifth and sixth decades of life, which was also reported by Chen, and emphasized by Milchgrub et al. in their case series report. The latter authors standardized the lesion and named it hyalinizing clear cell carcinoma. These prevalence data were similar to those described

later in several case series articles and review articles such as the one published by Yang et al. These authors reviewed 155 cases and identified a mean age of 57 years, and a majority of cases occurring in females. Our study presented a majority of cases in women (81.81%) with a mean age of 56.42 years, thus corroborating findings from these studies. An interesting finding in our study was the high frequency of white patients [1, 4, 21].

As to location, this neoplasm rarely occurs in major salivary glands; it is almost exclusive of minor salivary glands. Mostly the palate is reported as the main location followed by the tongue base (Bilodeau et al. (11) and Solar, Schmidt and Jordan (17). However, other studies such as the one of; Albergotti et al. (6) did not observe a predilection for any site. In our cases, palate and buccal mucosa were equally affected. In the literature, however, buccal mucosa is not described as a frequent location for clear cell carcinoma. None of our cases occurred in major salivary glands, which is corroborated by the findings in the literature [6, 21, 23, 24].

Other regions of the respiratory and upper part of digestive tracts are also affected by this lesion, such as the paranasal sinus, as described by Lan et al., Alali et al. and Zhao et al. In our study, only one case was in the oropharynx, which is a rare site of occurrence. Another observation made by the authors regards the clinical presentation of the neoplasm, which frequently presents as a painless mass and firm to palpation (which was also observed in most of our cases, as described in the patients` files). Probably the “less aggressive” behavior often causes patients to seek treatment at later stages of the disease. [5, 6, 21, 24–29]

Recurrence of this neoplasm is low, ranging from 12 to 19%, and lymph node metastasis is also low, occurring in about 5 to 25% of cases, thus suggesting that this lesion has low aggressiveness and good response to surgery. In literature reviews of case reports and case series, we observed few deaths related to this neoplasm, as described by Hernandez et al. who reported a 5-year survival in 89% of cases and a 10-year survival in 83%. Considering only deaths caused by cancer, the survival rate percentage rises to 97%. In our study, we could not make this relationship due to scarcity of data, since we are a diagnostic service only. However, we received information that one of the cases had a lymph node metastasis two years after the excision of the initial lesion. In the literature, recurrence and metastasis occur late in most cases. Yang et al., observed a mean recurrence period of 47.7 months, and Daniele et al. (3) reported 10 cases with recurrence after 5 years and 1 case after 24 years. Regarding distant metastasis, the most common site was the lung [3, 5, 8, 21, 24].

The presence of hyalinization, as described by Milchgrub et al., represents a controversy among authors. Ellis (15) has already criticized this nomenclature for reporting that hyalinization was not present in all cases, and Bilodeau et al. also reported the presence of hyalinization in only 64.7% of the cases, noting that sometimes there was only a thin fibrocellular cord. Daniele et al. and Hernandez et al. considered that all characteristics were variations of hyalinization, ranging from small fibrous septa to large sclerotic bands of hyaline material. Hyalinization was clearly observed in 9/11 of our cases, thus demonstrating that hyalinization is not always present in this lesion. Another justification for not having hyalinization in

all our cases is the amount of material that was sent for analysis; most of the time they were incisional biopsies, as in one of the cases in which we observed a descriptive diagnosis only, because there was a very scarce amount of tissue [1, 3, 4, 8, 23, 30].

The presence of polygonal clear cells arranged in cords, nests, and islands, with addition of eosinophilic cells around, is the main characteristic of this neoplasm, present in all cases, thus representing a consensus for all the authors studied. Neural infiltration was a common finding in the lesions, ranging from 33.33 to 47%, followed by necrosis with 6.66 to 18.75%. Mitosis and lymphovascular invasion were rarely described. Our study showed that 6/11of the cases had neural invasion and 2/11 had necrosis, signaling that neural infiltration is a very common finding. However, it is noteworthy that our cases are mostly incisional biopsies. Despite being very present, neural invasion has no connection with the prognosis of this neoplasm. All cases were positive for PAS nonresistant to diastase, showing it to be a glycogen-rich lesion and justifying why this neoplasm has already been called glycogen-rich clear cell carcinoma. [1, 6, 13, 21, 23–25, 31]

There are neoplasms of different origins that present clear cells, thus resulting in a need for a correct diagnosis, once each one of them show a different biological behavior and sometimes different treatments are required.

Among the lesions that originate in the salivary gland and may present clear cells, one can mention mucoepidermoid carcinoma, epithelial-myoepithelial carcinoma, myoepithelioma, myoepithelial carcinoma, acinc cell carcinoma, etc. Also, metastasis from renal clear cell carcinoma may occur in salivary glands. Odontogenic carcinomas rich in clear cells can also simulate a salivary gland clear cell carcinoma occurring inside the bones. However, the use of immunohistochemical panels usually is useful in differentiating among them. In our immunohistochemical panel, all clear cell carcinomas of salivary glands were negative for S-100 and SMA, as already reported in the literature, showing that they did not present myoepithelial cells. The positivity of CK7 and/or14, and negativity to S100 protein and SMA confirmed the absence of myoepithelial cells. These findings helped to differentiate clear cell carcinoma from epithelial-myoepithelial carcinoma, clear cell myoepithelial carcinoma or myoepithelioma. The presence of mucin in mucoepidermoid carcinomas is usually sufficient to differentiate it from PAS-diastasis negative clear cell carcinoma, as well as from acinic cell carcinoma with a predominance of clear cells. When there is mucin in clear cell carcinomas, it is very specifically located. [4, 13, 21, 23–25, 31]

Molecular tests as FISH and PCR have been recently employed as a tool to aid in the diagnosis of clear cell carcinoma. In the first one, the rearrangement of genes or fusion gene are observed, and in the second only the fusion gene can be detected. For instance, in mucoepidermoid carcinoma, the rearrangement of MAML2 gene is the most frequent and related to a better prognosis for this lesion. EWSR1-ATF1 fusion gene present in clear cell carcinoma was first reported by Antonescu et al. [10] that showed the rearrangement of the EWSR1 and ATF1 genes using FISH test, and they found the rearrangement in more than 80% of clear cell carcinomas. In addition, they performed a PCR counter-test

in two lesions, characterizing the EWSR1-ATF1 fusion gene. This fusion gene is not exclusive of this neoplasm, as it is also present in angio fibrous histiocytoma, pulmonary clear cell carcinoma and clear cell odontogenic carcinoma. However, none of these lesions originate from the salivary glands. [8, 14, 15, 19]

Bilodeau et al. carried out the same molecular experiment with both clear cell carcinoma of the salivary gland and clear cell odontogenic carcinoma, since the two lesions look similar from a histological standpoint and immunohistochemical profile. They also observed the presence of EWSR1-ATF1 fusion gene in more than 90% of the clear cell odontogenic carcinomas, which justifies the thought of some authors that the latter lesion would be a central clear cell carcinoma. However, it was observed by Kujiraoka et al., through cell culture of clear cell carcinoma of odontogenic origin and by Bilodeau et al., that clear cell odontogenic carcinomas have cells with nuclei arranged in palisade on its periphery, which favoring an odontogenic origin. It is believed that other fusion genes may be present in clear cell carcinomas as reported by Chapman et. al. These authors demonstrated the presence of EWSR1-CREW in three neoplasms diagnosed as clear cell carcinoma. More studies, however, need to be carried out to strengthen this hypothesis. For now, WHO only recognizes EWSR1-ATF1 expression for the clear cell carcinoma. In our study, we observed the presence of this fusion gene in 6/9 of the cases, indicating that this test may help in the diagnosis of this lesion. Our positivity rate for the EWSR1-ATF1 gene was a little lower than most studies, and we believe that two factors influenced our results: the amount of material available for the analysis, and the conservation of this material. It is known that PCR test is more specific, and more sensitive than FISH test [20, 23, 32, 33].

Since the finding of this gene, many authors have performed FISH test for the rearrangement of the EWSR1 gene to aid in the diagnosis, as it is a simple test to perform and does not depend so much on the amount of material and storage conditions. As a disadvantage, this test has a higher cost. Hsieh et al. reported that 3 neoplasms with an initial diagnosis of mucoepidermoid carcinoma were reclassified as clear cell carcinoma after FISH test turned out positive for EWSR1 gene rearrangement. This reclassification also considered the histological and immunohistochemical aspects. In our cases, we also reclassified some cases after reviewing their histological and immunohistochemical aspects. The rearrangement for the isolated EWSR1 gene does not add much to the diagnosis of this lesion, as this oncogene is very common in other lesions such as mucoepidermoid carcinoma and epithelial-myoepithelial carcinoma. Thus, both FISH test for ATF1 gene and the immunohistochemical profile that characterizes this lesion should be performed together [11, 20].

When PCR is performed to detect the EWSR1-ATF1 fusion gene (which is the characteristic gene of the lesion), the positivity of this test can be considered as a definitive factor for the diagnosis of this lesion, together with histological and immunohistochemical factors. However, this test was not the most used one in other studies, and when it was used, a small number of cases (2 or 3) was assessed, as this is a more sensitive exam and more difficult to perform. In our study, we aimed to evaluate, by PCR, the possible detection of the EWSR1-ATF1 gene in tissue that had been embedded in paraffin for over 10 years. Real-time PCR tests determined that 66.66% of our clear cell carcinoma cases had EWSR1-ATF1

fusion gene, indicating that this test can aid the diagnosis. As an addendum, most positive cases were observed in more recent cases, thus demonstrating the difficulty of testing in tissues embedded in paraffin for longer periods of time. Therefore, more recently, some authors have been recommending that the diagnosis should only be made with histological and immunohistochemical tests and that molecular tests should only be done when there is any doubt regarding the diagnosis [12, 14, 22, 24, 35].

Conclusion

- In our sample, clear cell carcinoma was more prevalent in women aged between 50 and 60 years, in minor salivary glands and with a predilection for the palate.
- Histological aspects and immunohistochemical expression, especially of cytokeratin 7 and p63 protein, associated with negativity for S100 and smooth muscle actin, in most cases, may be sufficient to confirm this diagnosis, as it is a lesion with a single type of cells.
- Hyalinization is not always present in the lesion and neural invasion is very common in clear cell carcinoma.
- Molecular tests can aid diagnosis. However, immunohistochemical aspects cannot be ignored, as EWSR1-ATF1 gene is not present in 100% of cases. In our study, the PCR result for the EWSR1-ATF1 gene was positive in 66.66% of the cases.
- Clinical and radiographic features are essential for differential diagnosis between odontogenic clear cell carcinoma and clear cell carcinoma of salivary gland, once they are histologically and immunohistochemically similar.

References

1. - Chen KT. Clear cell carcinoma of the salivary gland. Human pathol. 1983;14(1):91–3.
2. - Kauzman A, Tabet JC, Stiharu TI. Hyalinizing clear cell carcinoma: a case report and review of the literature. Oral Surg, Oral Med, Oral Pathol, Oral Radiol, and Endod. 2011;112(1):e26-34. doi: 10.1016/j.tripleo.2011.02.041.
3. - Daniele L, Nikolarakos D, Keenan J, Schaefer N, Lam AK. Clear cell carcinoma, not otherwise specified/hyalinising clear cell carcinoma of the salivary gland: The current nomenclature, clinical/pathological characteristics and management. Crit Rev Oncol Hematol. 2016;102:55–64. doi: 10.1016/j.critrevonc.2016.03.018.
4. - Milchgrub S, Gnepp DR, Vuitch F, Delgado R, Albores-Saavedra J. Hyalinizing clear cell carcinoma of salivary gland. Am J of Surg Pathol. 1994;18(1):74–82.
5. - Felix A, Rosa JC, Nunes JF, Fonseca I, Cidadao A, Soares J. Hyalinizing clear cell carcinoma of salivary glands: a study of extracellular matrix. Oral Oncol. 2002;38(4):364–8.
6. - Albergotti WG, Bilodeau EA, Byrd JK, Mims MM, Lee S, Kim S. Hyalinizing clear cell carcinoma of the head and neck: Case series and update. Head Neck. 2016;38(3):426–33. doi: 10.1002/hed.23902.

7. - Weinreb I. Hyalinizing clear cell carcinoma of salivary gland: a review and update. Head Neck pathol. 2013;7 Suppl 1:S20-9. doi: 10.1007/s12105-013-0466-8.
8. - Hernandez-Prera JC, Kwan R, Tripodi J, Chiosea S, Cordon-Cardo C, Najfeld V, et al. Reappraising hyalinizing clear cell carcinoma: A population-based study with molecular confirmation. Head Neck. 2017;39(3):503–11. doi: 10.1002/hed.24637.
9. - Shah AA, LeGallo RD, van Zante A, Frierson HF, Jr., Mills SE, Berean KW, et al. EWSR1 genetic rearrangements in salivary gland tumors: a specific and very common feature of hyalinizing clear cell carcinoma. Am J Surg Pathol. 2013 Apr;37(4):571–8. doi: 10.1097/PAS.0b013e3182772a15.
10. - Sun Z. J. ZYF, Zhang L., Zhang W. F.,Chen X. M.,He S. G. Hyalinizing clear cell carcinoma in minor salivary glands of maxillary tuberosity. Oral Oncology EXTRA. 2005:306–10.
11. - Skalova A, Weinreb I, Hyrcza M, Simpson RH, Laco J, Agaimy A, et al. Clear cell myoepithelial carcinoma of salivary glands showing EWSR1 rearrangement: molecular analysis of 94 salivary gland carcinomas with prominent clear cell component. Am J Surg Pathol. 2015 Mar;39(3):338–48. doi: 10.1097/PAS.0000000000000364.
12. - Yin LX, Ha PK. Genetic alterations in salivary gland cancers. Cancer. 2016 Jun 15;122(12):1822–31. doi: 10.1002/cncr.29890.
13. - Seethala RR, Stenman G. Update from the 4th Edition of the World Health Organization Classification of Head and Neck Tumours: Tumors of the Salivary Gland. Head Neck Pathol. 2017;11(1):55–67. doi: 10.1007/s12105-017-0795-0.
14. - Antonescu CR, Katabi N, Zhang L, Sung YS, Seethala RR, Jordan RC, et al. EWSR1-ATF1 fusion is a novel and consistent finding in hyalinizing clear-cell carcinoma of salivary gland. Genes Chromosomes Cancer. 2011;50(7):559–70. doi: 10.1002/gcc.20881.
15. - Brandwein-Gensler M, Wei S. Envisioning the next WHO head and neck classification. Head Neck Pathol. 2014;8(1):1–15. doi: 10.1007/s12105-014-0529-5.
16. - Weinreb I. Translocation-associated salivary gland tumors: a review and update. Adv Anat Pathol. 2013 Nov;20(6):367–77. doi: 10.1097/PAP.0b013e3182a92cc3.
17. - Tanguay J, Weinreb I. What the EWSR1-ATF1 fusion has taught us about hyalinizing clear cell carcinoma. Head Neck Pathol. 2013 Mar;7(1):28–34. doi: 10.1007/s12105-013-0427-2.
18. - Simpson RH, Skalova A, Di Palma S, Leivo I. Recent advances in the diagnostic pathology of salivary carcinomas. Virchows Arch. 2014 Oct;465(4):371–84. doi: 10.1007/s00428-014-1639-x.
19. - Fonseca FP, Sena Filho M, Altemani A, Speight PM, Vargas PA. Molecular signature of salivary gland tumors: potential use as diagnostic and prognostic marker. J Oral Pathol Med. 2016 Feb;45(2):101–10. doi: 10.1111/jop.12329.
20. - Chapman E, Skalova A, Ptakova N, Martinek P, Goytain A, Tucker T, et al. Molecular Profiling of Hyalinizing Clear Cell Carcinomas Revealed a Subset of Tumors Harboring a Novel EWSR1-CREM Fusion: Report of 3 Cases. Am J Surg Pathol. 2018 Sep;42(9):1182–1189. doi: 10.1097/PAS.0000000000001114.

21. - Yang XH, Liu L, Shi YY, Hu YJ, Hu QG, Zhang P. Hyalinizing clear cell carcinoma of salivary gland origin in the head and neck: clinical and histopathological analysis. Int J Oral Maxillofac Surg. 2018 Jun;47(6):692–698. doi: 10.1016/j.ijom.2017.11.004.
22. - Nakano T, Yamamoto H, Nishijima T, Tamiya S, Shiratsuchi H, Nakashima T, et al. Hyalinizing clear cell carcinoma with EWSR1-ATF1 fusion gene: report of three cases with molecular analyses. Virchows Arch. 2015 Jan;466(1):37–43. doi: 10.1007/s00428-014-1676-5.
23. - Bilodeau EA, Hoschar AP, Barnes EL, Hunt JL, Seethala RR. Clear cell carcinoma and clear cell odontogenic carcinoma: a comparative clinicopathologic and immunohistochemical study. Head Neck Pathol. 2011;5(2):101–7. doi: 10.1007/s12105-011-0244-4.
24. - Solar AA, Schmidt BL, Jordan RC. Hyalinizing clear cell carcinoma: case series and comprehensive review of the literature. Cancer. 2009 Jan 1;115(1):75–83. doi: 10.1002/cncr.23974.
25. - Thway K, Fisher C. Tumors with EWSR1-CREB1 and EWSR1-ATF1 fusions: the current status. Am J Surg Pathol. 2012 Jul;36(7):e1-e11. doi: 10.1097/PAS.0b013e31825485c5.
26. - Zhao W, Yang L, Wang L, Zuo W, Yuan S, Yu J, et al. Primary clear cell carcinoma of nasal cavity: report of six cases and review of literature. Int J Clin Exp Med. 2014 Dec 15;7(12):5469–76. eCollection 2014.
27. - Lan J, Huang SC, Chen YH, Chen WC, Jin YT, Lu YC, et al. Primary paranasal sinus clear cell carcinoma with EWSR1-ATF1 fusion: report of 2 molecularly confirmed cases exhibiting unique histopathology. Hum Pathol. 2017 May;63:139–143. doi: 10.1016/j.humpath.2016.09.036. Epub 2016 Dec 1
28. - AlAli BM, Alyousef MJ, Kamel AS, Al Hamad MA, Al-Bar MH, Algowiez RM. Primary paranasal sinus hyalinizing clear cell carcinoma: a case report. Diagn Pathol. 2017 Sep 25;12(1):70. doi: 10.1186/s13000-017-0659-7.
29. - Zhao YN, Wang X, Liang FH, Zhang WJ, Song XT. Hyalinizing clear cell carcinoma of salivary glands: A retrospective study focused on uncommon morphology, immunohistochemistry, and detection of gene fusion using fluorescence in situ hybridization. Pathol Res Pract. 2018 Mar;214(3):380–384. doi: 10.1016/j.prp.2017.12.021.
30. - Ellis GL. Clear cell neoplasms in salivary glands: clearly a diagnostic challenge. Annals of diagnostic pathology. Ann Diagn Pathol. 1998 Feb;2(1):61–78.
31. - Pujary K, Rangarajan S, Nayak DR, Balakrishnan R, Ramakrishnan V. Hyalinizing clear cell carcinoma of the base of tongue. Int J Oral and Maxillofac Surg. 2008;37(1):93–6.
32. - Bilodeau EA, Weinreb I, Antonescu CR, Zhang L, Dacic S, Muller S, et al. Clear cell odontogenic carcinomas show EWSR1 rearrangements: a novel finding and a biological link to salivary clear cell carcinomas. Am J Surg Pathol. 2013 Jul;37(7):1001–5. doi: 10.1097/PAS.0b013e31828a6727.
33. - Kujiraoka S, Tsunematsu T, Sato Y, Yoshida M, Ishikawa A, Tohyama R, et al. Establishment and characterization of a clear cell odontogenic carcinoma cell line with EWSR1-ATF1 fusion gene. Oral Oncol. 2017 Jun;69:46–55. doi:10.1016/j.oraloncology.2017.04.003.

34. - Hsieh MS, Wang H, Lee YH, Ko JY, Chang YL. Reevaluation of MAML2 fusion-negative mucoepidermoid carcinoma: a subgroup being actually hyalinizing clear cell carcinoma of the salivary gland with EWSR1 translocation. Human Pathol. 2017 Mar;61:9–18. doi: 10.1016/j.humpath.2016.06.029.
35. - Skalova A, Stenman G, Simpson RHW, Hellquist H, Slouka D, Svoboda T, et al. The Role of Molecular Testing in the Differential Diagnosis of Salivary Gland Carcinomas. Am J Surg Pathol. 2018 Feb;42(2):e11-e27. doi:10.1097/PAS.0000000000000980

Table 1

Table 1 is available in the Supplementary Files section.

Figures

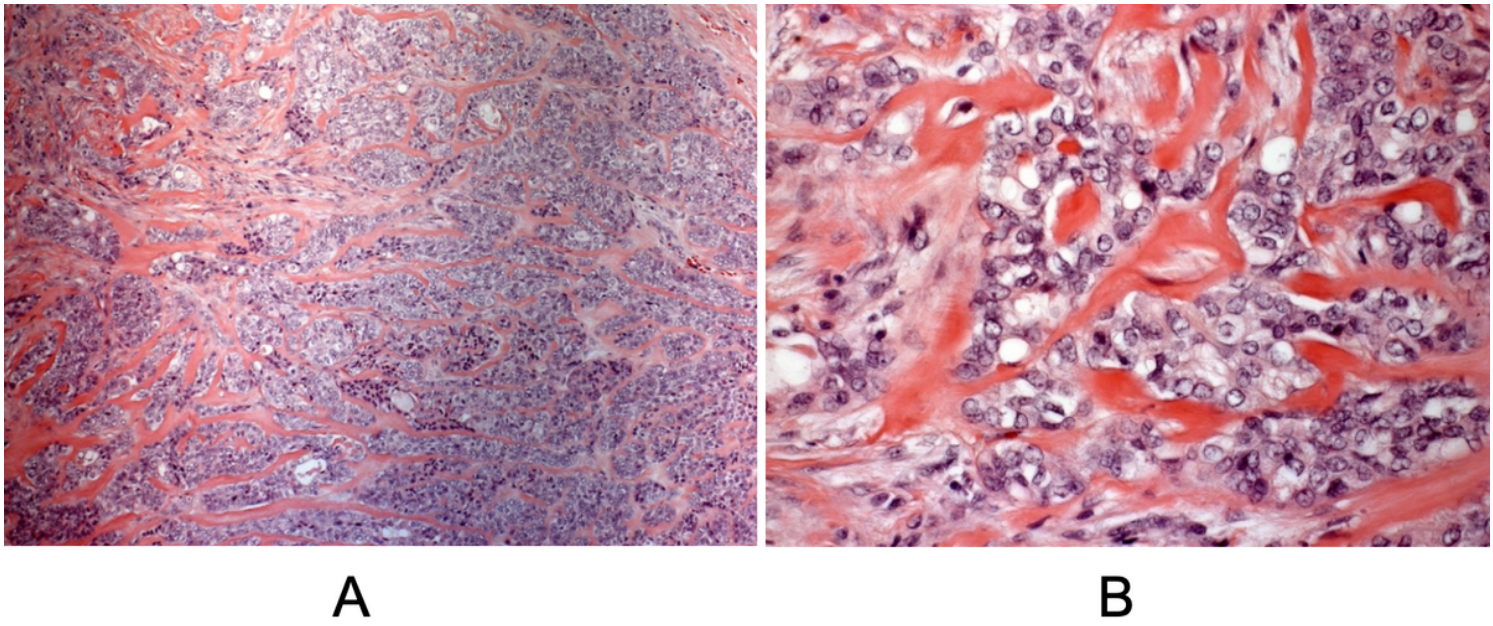

Figure 1

Histological aspects in Hematoxylin Eosin a) 10x magnification b) 40x magnification showing clear cells in hyaline stroma.

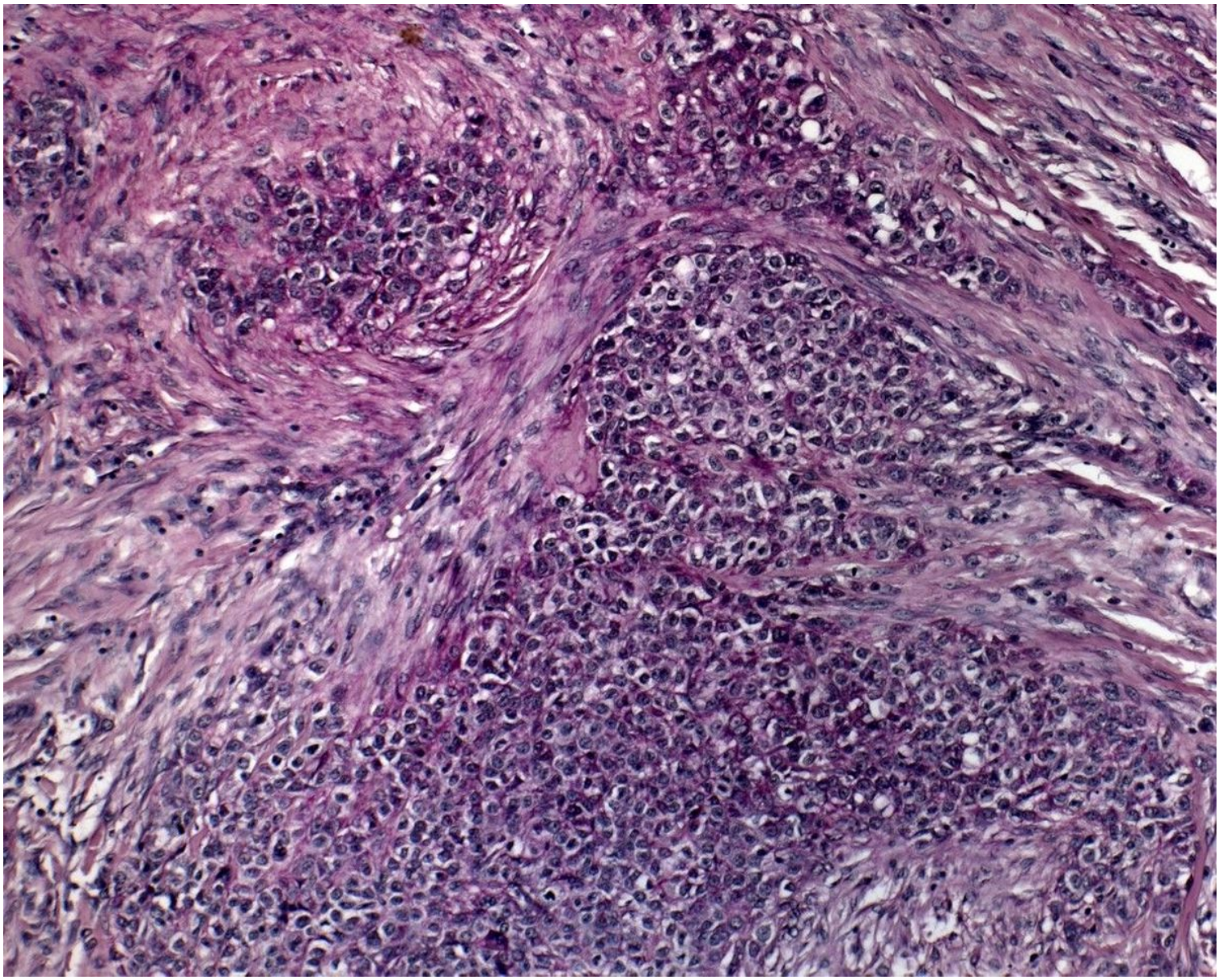

Figure 2

PAS no diastasis in histological fragment.

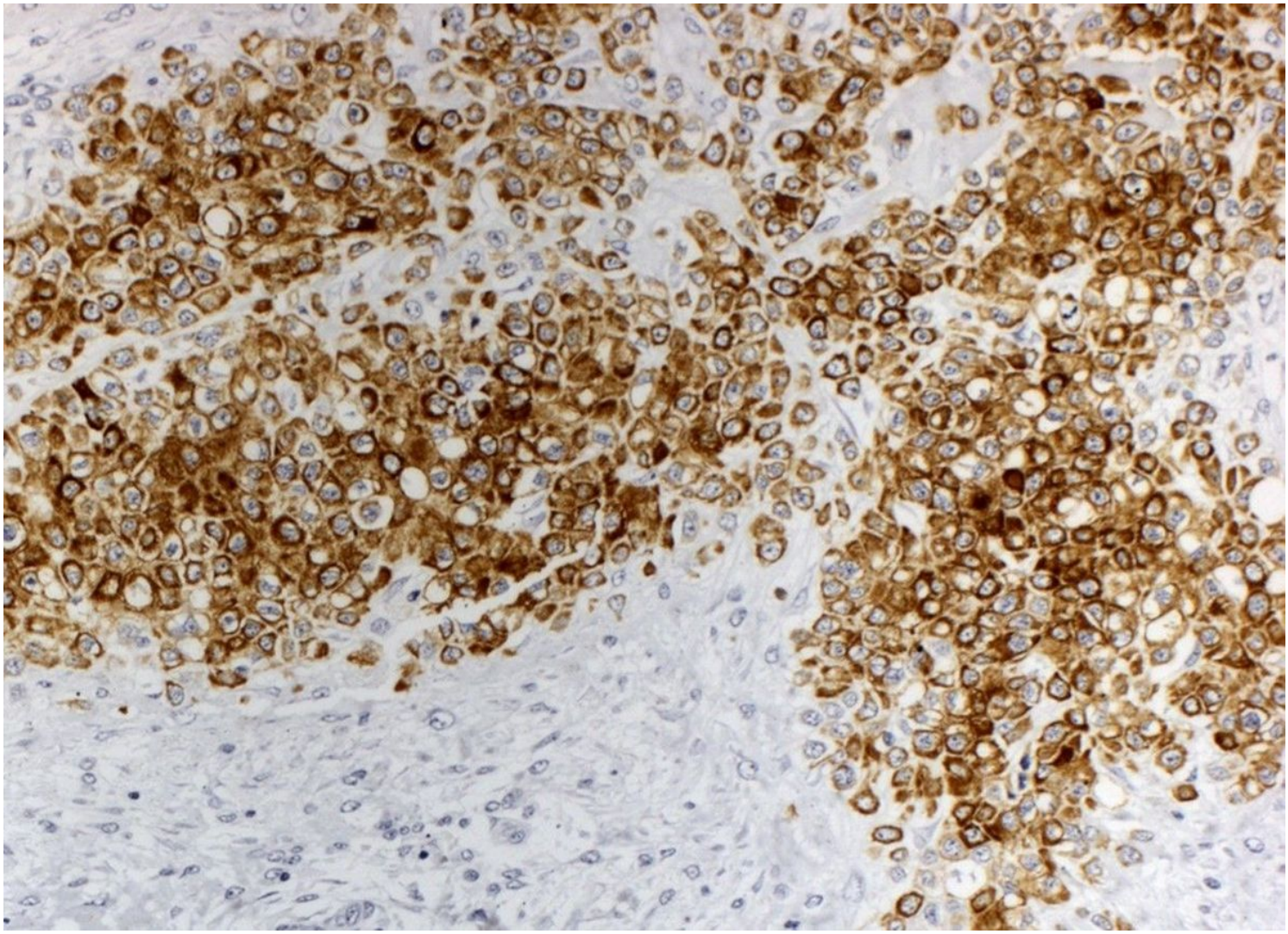

Figure 3

Cytokeratin 7 positive in histological fragment.

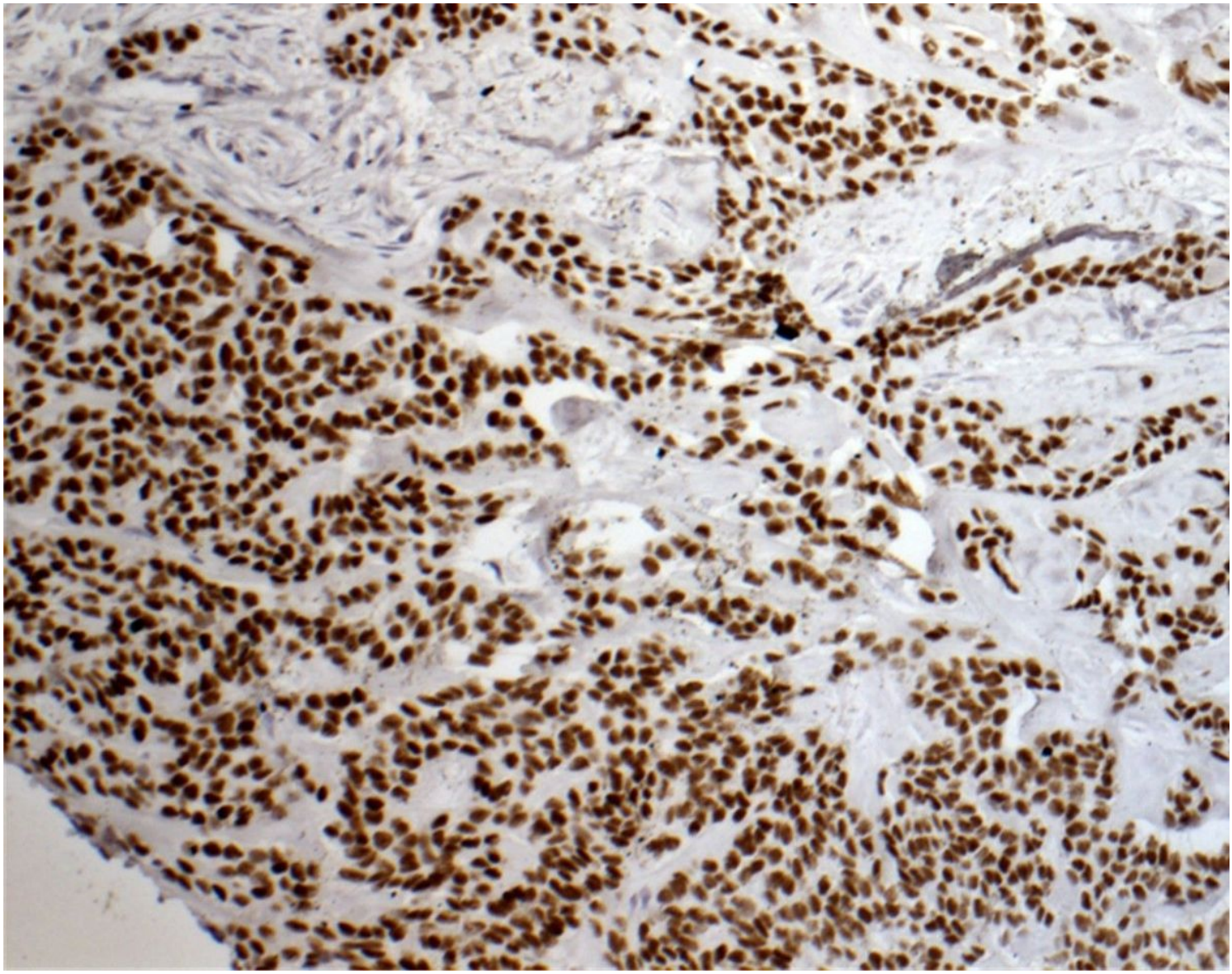

Figure 4

p63 positive in histological fragment.

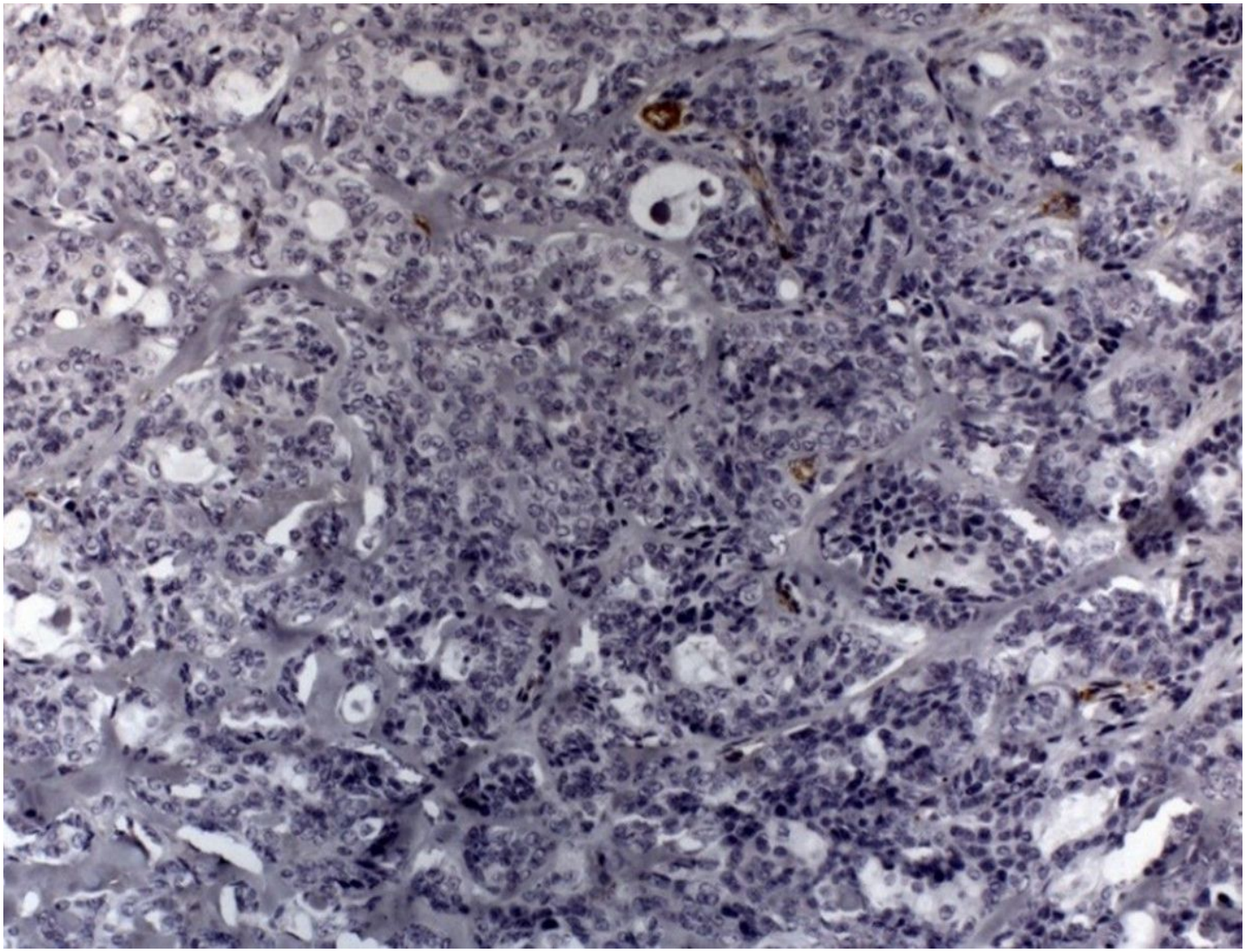

Figure 5

Smooth muscle actin negative in histological fragment.

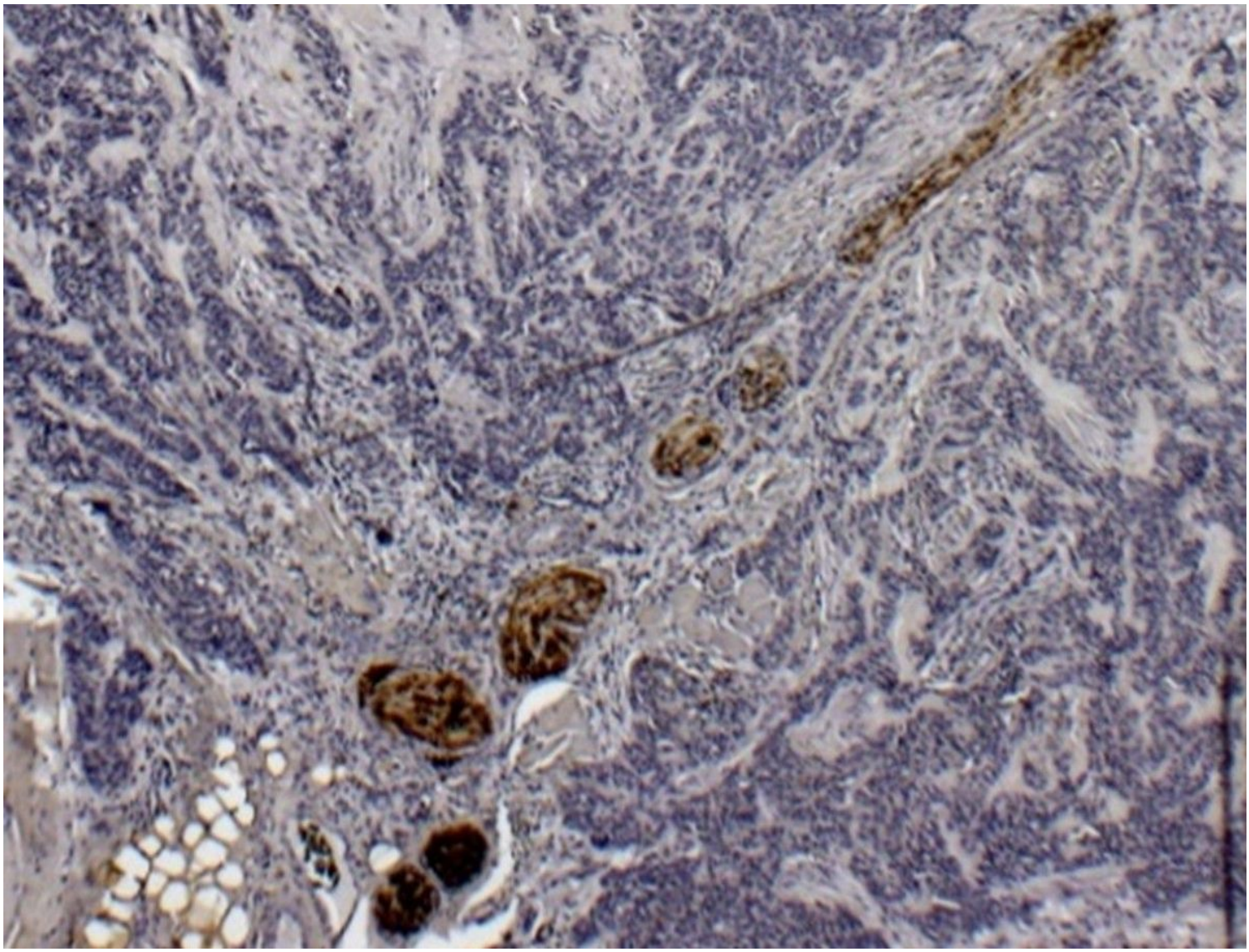

Figure 6

S-100 negative in the lesion and positive in neural tissue, demonstrating neural infiltration of the lesion.

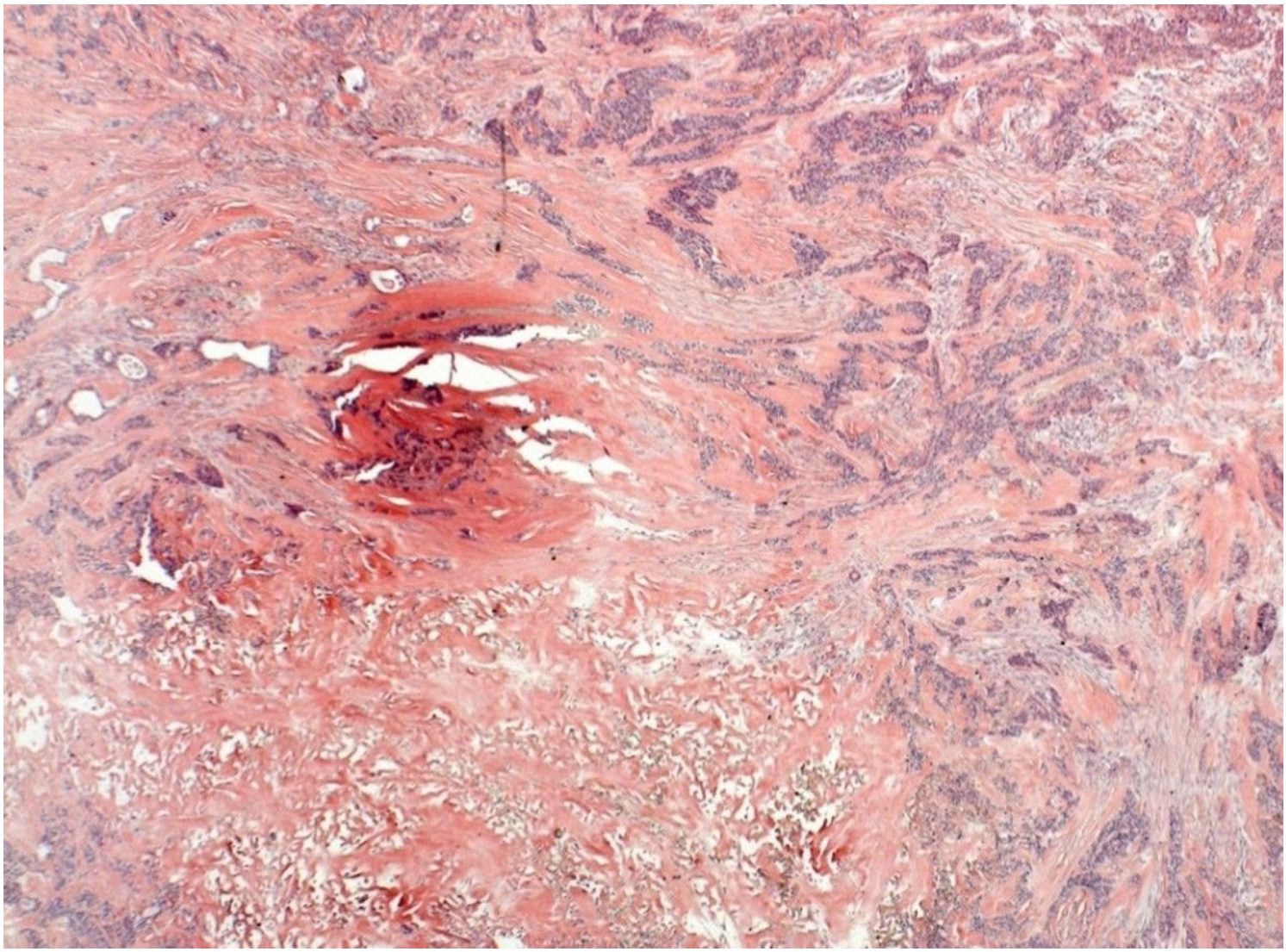

Figure 7

Area of necrosis within the lesion.

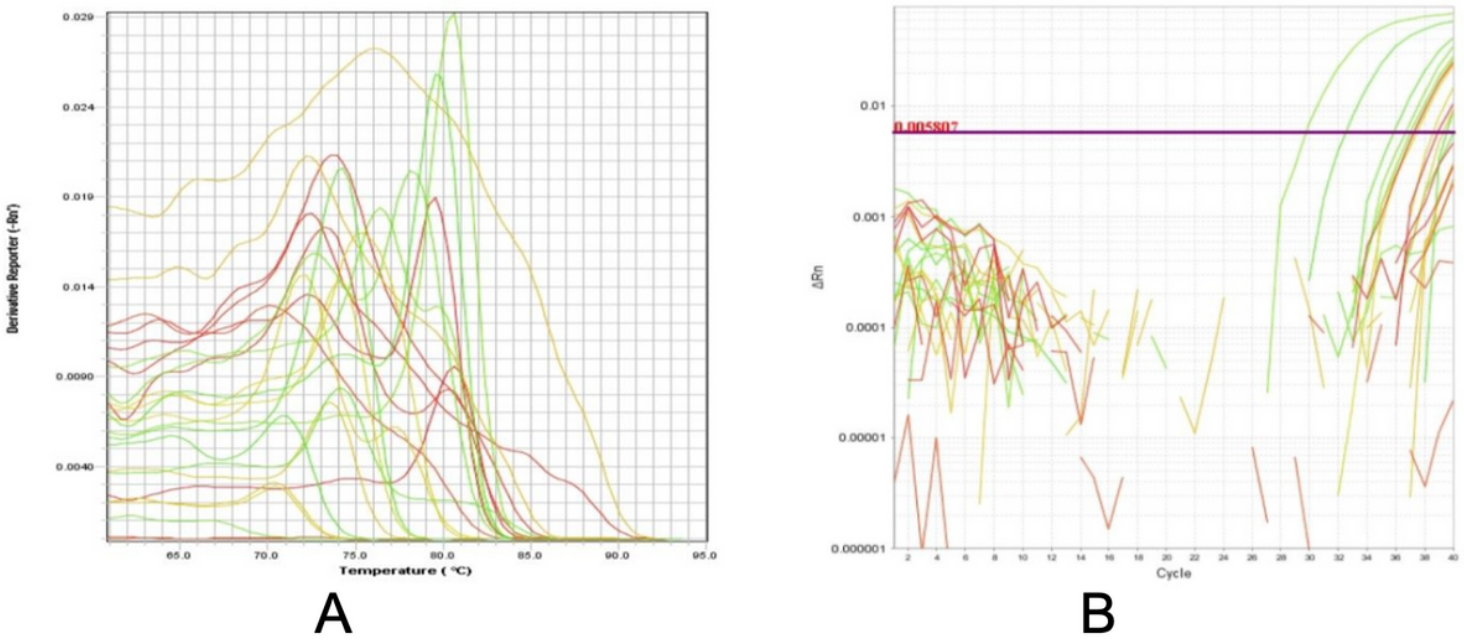

Figure 8

Real-time PCR, a) melting curve showing the normality profile of the PCR and b) plot graph showing amplifications.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- tables.docx