Demonstration of microRNA using in situ hybridisation on formalin fixed paraffin wax samples using conventional oligonucleotide probes: A comparison with the use of locked nucleic acid probes

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Manuscript

Title:

Demonstration of microRNA using *in situ* hybridisation on formalin fixed paraffin wax samples using conventional oligonucleotide probes: A comparison with the use of locked nucleic acid probes.

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Disclosure statement

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Abstract

Background: MicroRNAs (miRNA) regulate the translation of mRNA during gene expression and investigations have highlighted their importance in pathophysiology. qRT-PCR is currently the gold standard method for detecting changes in miRNA expression. However, when used on heterogeneous samples, it cannot identify individual cell types harbouring miRNAs. For this, *in situ* hybridisation (ISH) can be used. ISH methods using locked nucleic acid (LNA) probes have been shown to give reliable results in formalin fixed paraffin embedded (FFPE) samples. In this study their use has been directly compared with conventional oligonucleotide probes (COP) for ISH.

Methods: FFPE samples of colorectal adenocarcinoma, squamous carcinoma of lung and cases of invasive breast carcinoma were used to evaluate COP and LNA methods for the demonstration of miR-126 and miR-205. To demonstrate the utility of the COP method demonstration of miR-21 in 19 Gleason stage 7 prostate biopsy FFPE tissues was also undertaken. The demonstration of miR-21 by ISH in high and low expressing prostate cancer cell lines was also compared with qRT-PCR.

Results: Similar results were obtained using the COP and LNA ISH methods for the demonstration of miR-126 and miR-205. miR-21 was successfully demonstrated in the prostate cancer samples by COP ISH and expression levels of the miRNA demonstrated in the cell lines corresponded with qRT-PCR.

Conclusion: This study has shown that simplification of ISH protocols by the use of COPs provides equivalent results to the use of LNA methods and it can be used to precisely identify cells in which miRNAs are expressed.

Introduction

MicroRNAs (miRNA) represent a non-coding family of 21-25 nucleotide long RNAs that regulate translation during gene expression [1,2]. A single miRNA can target hundreds of individual mRNAs and they have been shown to play an important role in pathophysiology [3,4]. They regulate cell proliferation, apoptosis, cell differentiation, cell migration and cell cycle control; processes that are linked to cancer [5,6].

The expression levels of miRNAs have been extensively studied using molecular methods such as qRT-PCR on homogenate sample preparations. These methods provide for the rapid and quantitative assessment of miRNAs and determination of their altered expression in pathological states. Applied to homogenous cell populations they provide a reliable indicator of alteration of miRNA expression. However, in situations where the cell population is heterogenous, as in tissue samples, then they provide only an overall indicator of expression, but cannot identify the alteration of miRNA levels in individual cell types. The latter has been shown to be important in the clarifying potential conclusions for the role of individual miRNAs in disease. In particular, the assignment of miR-143 and miR-145 as inhibitors of tumourigenesis in colonic cancer was shown, at the cellular level, to be associated with changes in mesenchymal derived smooth muscle and myofibroblast cells involved in repair processes instead [7]. In this study *in situ* hybridisation (ISH) was used to clarify the alterations in miRNA expression at the cellular level. In a commentary on this work Kent, et al [8] strongly advocated that ISH should always be used alongside techniques that provide an overall indication of miRNA expression in order to assist in arriving at an accurate determination of the precise cause/s of pathological change.

miRNAs have been successfully demonstrated by ISH using locked nucleic acid probes (LNA™) applied to formalin fixed paraffin embedded (FFPE) tissue sections [9]. It has been proposed that due to their high hybridisation efficiency that this type of probe is required to ensure optimal hybridisation to the short miRNA sequences.

With the purpose of providing an alternative ISH method, conventional oligonucleotide probes (COP) were used to demonstrate miR-126 and miR-205 in FFPE sections of colorectal carcinoma (CRC), squamous carcinoma (SCC) of lung and cases of invasive breast carcinoma (IBC). When compared with the use of LNA™ probes of identical sequence similar results were obtained. To further explore the use of COP ISH miR-21 was assessed in 19 Gleason stage 7 prostate core biopsy FFPE tissues. COP ISH and qRT-PCR was also undertaken on high and low expressing prostate cancer cell lines with a positive consensus in results being demonstrated.

Materials and methods

FFPE sections of CRC from surgical resections and core biopsy SCC of lung were obtained from Queen's London Hospital, London. FFPE sections from 20 Gleason stage 7 prostate core biopsies were obtained from the University College London Cancer Institute, Biobank and Pathology Core Facility Service, London. All were transferred and held in the University of Westminster Biobank in compliance with Human Tissue Authority regulations. FFPE IBC blocks held in the University of Westminster, Against Breast Cancer Tissue bank were also used. Sections from these surgical resection cases were cut at 4 µm. All sections were mounted on SuperFrost plus slides.

Prostate cancer cell lines, LNCaP and PC3 were obtained from the American Type Culture Collection (Manassas, USA). These were maintained in Roswell Park Memorial Institute (RPMI) 1640 (Gibco-Life Technologies, UK) with 10% (v/v) heat-inactivated foetal bovine serum (Pan Biotech, Germany) and penicillin-streptomycin (10,000 units penicillin / ml, 10 mg streptomycin / ml - Pan Biotech, Germany) at 37 °C in a humidified 5 % CO_2 incubator.

For the preparation of FFPE cell blocks for ISH harvested cells were collected into phosphate-buffered saline (PBS) and centrifuged for 5 mins at 2700 g twice and the pellets re-suspended in 100 µl of PBS. 10 ml of neutral buffered formalin (NBF) was added and the cells were fixed for 30 mins. The cells were then washed and centrifuged twice in PBS as before. 10% gelatine dissolved in tris buffered saline was prepared and 0.5 ml added to the cell pellets. After hardening, each cell block was re-fixed in NFB for 30 mins. Following washing in PBS, the blocks were held overnight in 70 % alcohol at 4 °C. They were then immersed in 100 % alcohol at ambient temperature for 45 mins then in the same for 60 mins, two changes of xylene for 45 mins each, immersed in paraffin wax and then embedded in the same. Sections from the cell blocks were cut at 5 µm and mounted on SuperFrost plus slides.

A ready to use fluorescein labelled Poly d (T) probe (Leica, POLYDT, UK) was used to assess the overall preservation of RNA in each sample and to determine the optimal Proteinase K concentration required to unmask miRNA.

The COPs for demonstration of specific miRNA species were obtained from Integrated DNA Technologies, BVBA, Belgium. The sequences for miR-21, miR-126, miR-205 and scrambled oligonucleotides were identical to those used by Jørgensen, et al [9], see Table 1. The probes were labelled with digoxigenin (DIG) inserted as an NHS ester 3' and 5' during synthesis and HPLC purified before delivery. The DIG labelled oligonucleotides were reconstituted in 1 x TE pH 8.0 aliquoted and stored at -20 °C until used.

For comparative purposes LNA™ Digoxigenin labelled miR-126 probe together with pre-treatment and detection reagents (90005) and LNA™ Digoxigenin labelled miR-205 probe, (18099-15) were obtained from Exiqon, Denmark.

All cases for *in situ* hybridisation were initially screened for the presence of mRNA and determination of optimal Proteinase K pre-treatment using the poly d (T) probe. Subsequently samples were hybridised with the Digoxigenin labelled COP and /or LNA™ probes. The probe concentrations for the Digoxigenin labelled COPs ranged from 50 to 500 ng / ml, while those for the LNA™ probes were 40 nM. The ISH methodologies used are summarised in Table 2. Special care was taken to ensure an RNase-free environment. All glassware was sterilised to ensure RNase free activity. Gloves were worn at all times and RNase depleted water (DEPC water) was used in the preparation of solutions.

For qRT-PCR, RNA was extracted from cell lines using Trizol (Sigma, U.K.) and RNA concentration and purity were measured using a NanoDrop spectrophotometer at 260 nm and 280 nm. RNA was reverse-transcribed to cDNA using the qScript microRNA cDNA Synthesis Kit (Quantabio, UK.) according to the manufacturer's instructions. The resulting cDNA was used to assess the expression of miR-21, while U6-snRNA and hsa-let-7a-5p were used as a reference RNA for normalization of miRNA expression levels. The PerfeCTa SYBR® Green SuperMix (Quantabio, UK.) was used together with MystiCq microRNA qRT-PCR primers for miR-21 (hsa-miR-21-5p - MIRAP00047-250RXN - Sigma, UK). The sequences for

U6-snRNA primers were U6 forward, 5’-GCTTCGGCAGCACATATACTAAAAT-3’ and hsa-let-7a-5p forward 5’-CCGAGCTGAGGTAGTAGGTTGTATA-3’ together with reverse 5’-CGCTTCACGAATTTGCGTGTCAT-3’ for both. Thermocycling conditions were: initial denaturation at 95 °C for 2 mins, followed by 40 cycles of 95 °C for 5 secs, 60 °C for 15 secs, and terminating extension at 72 °C for 15 secs. Each run was repeated three times in triplicate.

Microscopic staining patterns were assessed qualitatively concentrating on identification of the cellular structures stained by the ISH probes. According to a standard method the intensity of staining was classified as weak, moderate or strong. For the prostate tissue samples the proportion of tumour glands in which staining was present was qualitatively determined.

The relative expression of miR-21 by qRT-PCR in the LNCaP and PC3 cell lines was normalized with U6 and let-7 expression using the comparative Cycle Threshold method [10]. The mean and standard deviation was then determined while t-Test and p values provided a comparator between the expression of miR-21 in the cell lines.

Results

COP ISH work up was undertaken using three cases each of CRC and SCC of lung together with two cases of IBC. The preservation of mRNA confirmed in all of the cases using the poly d (T) probe. The results were also used as a guide to determine the optimal Proteinase K pre-treatment for the cases. This was either 2 or 5 μg / ml.

The optimal probe concentration for miR-126 COP in the CRC and SCC of lung cases was 400 ng / ml. In these tissues strong staining of the endothelium of capillaries and blood vessels was observed (Fig 1a, 1b). In the IBC cases the optimal probe concentration was 500 ng / ml. In these cases endothelial staining was variably demonstrated and was supplemented by weak to moderate staining of the tumour islands (Fig 1e). In one case epidermis was present and this was also weakly stained for miR-126.

The optimal probe concentration for miR-205 COP in the CRC, SCC of lung and IBC cases was 500 ng / ml. In two of the CRC cases miR-205 was demonstrated in the tumour and uninvolved glandular epithelium (Fig 1c). The staining of the former was of weak to strong intensity, while in the latter strong staining was noted. In the remaining CRC case general staining of weak to moderate intensity was observed with no discrimination between tumour and stromal areas. Tumour islands and individual tumour cells were moderately to strongly stained in two cases of SCC of lung with the miR-205 COP (Fig 1d, 1g). In the remaining SCC of lung case no obvious tumour cells were present and staining for miR-205 was absent. Tumour islands in the IBC cases were stained at moderate to strong intensity. Epidermis, present in one case, was stained by the miR-205 COP at strong intensity.

The comparison of miR-126 ISH using COP and LNA™ probes was undertaken for miR-126 using the IBC cases. Endothelial and tumour staining was observed with both probe types in both cases. However, endothelial staining was more consistently demonstrated with the LNA™ probe. Staining of the tumour islands in one case with the LNA™ miR-126 probe was intense and obscured the staining of adjacent capillaries and small blood vessels (Fig 1f). In contrast the staining of the tumour islands in this case using COP ISH was weaker and endothelial staining in capillaries and small blood vessels clearly identifiable (Fig 1e). Epidermis, present in one case, was stained with miR-126 LNA™ at strong intensity and weak intensity with miR-126 COP.

Comparative ISH for miR-205 was undertaken using the two IBC cases and one case of SCC of lung. In all cases equivalent staining was observed using the COP and LNA™ ISH protocols. Tumour islands and epidermis were stained, respectively, at moderate and strong intensity in the IBC cases. Moderate to strong staining of the tumour islands and individual tumour cells in the SCC of lung was observed (Fig 1g, 1h).

A clear distinction was observed on miR-21 expression by qRT-PCR between the highly metastatic androgen independent PC3 and less metastatic LNCaP androgen sensitive cell lines. For the PC3 cells line the mean and standard deviation from the normalised expression levels was 13.7 and 3.6 respectively. For the LNCaP cell line the mean and standard deviation was 3.372 and 1.5 respectively. The t-Test value between the cell lines was 0.0023. In the PC3 cells miR-21 expression was 160-fold greater than that recorded for the LNCaP cells (n=3; p=0.002). This differential expression was mirrored in the COP ISH results (Fig 1i, 1j).

Of the 20 Gleason stage 7 cases one did not stain for mRNA using the poly d (T) probe. In the remaining 19 cases satisfactory staining was observed and these cases were taken forward for ISH using miR-21 and scrambled COP probes. The sections were pre-treated with Proteinase K at 5 or 10 ug / ml before hybridisation with the probes. Specific staining of the tumour glands at varying intensity and distribution was observed in 14 of the 19 cases, while in three cases non-specific staining of the tissue only was present and in the remaining two cases no staining was observed (Fig 1k, Table 3). No specific staining was recorded in sections hybridised with the scrambled COP probe.

Discussion

miRNAs have been demonstrated using a variety of ISH protocols. Thompson, et al [11] described a method using fluorescein labelled oligonucleotide probes that was complicated and took three days to perform. The technique was simplified by Nuovo, [12] and a one-day chromogenic technique using LNA™ probes was described by Jørgensen, et al [9].

The objective of this study was to establish if COPs could be used as an alternative to LNA™ probes for ISH to demonstrate miRNAs in FFPE preparations. Three miRNA targets were chosen to assess if this was possible, miR-21, miR-126 and miR-205. These were chosen as existing LNA™ ISH data was available [9] and expression profiles have been established using qRT-PCR methods in the tissues used in the present study.

miR-21, normally expressed in variety of haematopoietic cells, has been shown to be overexpressed in several tumour types and is considered to be an oncomiR [13,14]. miR-21 is associated with prostate cancer where *in vivo* models have shown that it can override androgen dependency [15] and promote metastatic growth [16]. In a meta-analysis of miRNAs and prostate cancer Song, et al [17] highlighted miR-21 as being associated with poor recurrence free survival. Combined ISH and immunohistochemical studies [18,19] have demonstrated localisation of miR-21 to prostatic epithelium and matched PTEN expression.

Present in endothelial cells miR-126 is important in the control of angiogenesis [20,21]. miR-126 has also been shown to be associated with cancer, with the levels being decreased in CRC [22,23], hepatocellular carcinoma [24] and non-small cell lung cancer [25].

miR-205 is expressed in normal squamous epithelia and in head and neck SCC [26,27]. An increase in miR-205 has been reported in cervical cancer tissue [28] and in oesophageal SCC cell lines [29]. Lebanony, et al [30] demonstrated miR-205 expression in SCC of lung to be higher than other forms of non-small cell lung cancer. Using LNA™ ISH Quesne, et al [31] reported the association of miR-205 with ductal breast carcinoma, while Wu, et al [32] reported suppression of cell growth and invasion by miR-205 in breast cancer.

The results of this study provide evidence that COP ISH can be used to demonstrate miRNAs in FFPE cell and tissue preparations. Furthermore, in terms of the known distribution of the three miRNAs studied, the results are in broad agreement with reports as summarised above. The strong staining of miR-126 in endothelium, miR-205 in normal epidermis and SCC of lung was as expected [20,21,26,27,28]. The staining of the tumour islands in IBC with the miR-205 also matches the expression profile of this miRNA for this cancer [31,32]. The differential expression of miR-205 in CRC with stronger staining of the miRNA in normal glandular epithelium compared with that in adjacent adenocarcinoma and the weak staining of miR-126 in the tumour islands of IBC illustrate the finesse of the ISH procedure and how this can add value to understanding expression levels obtained using qRT-PCR based methods.

The COP ISH method was compared with the LNA™ ISH procedure using probes of identical sequence in cases of IBC and SCC of lung. Endothelium was demonstrated more consistently using miR-126 LNA™ ISH than with COP ISH. The intensity of staining for this miRNA was also greater with the former probe in the tumour islands of an IBC case and in normal epidermis than in tissues hybridised using the COP ISH procedure. These results could be explained by the higher hybridisation efficiency of the LNA™ probe. However, the demonstration of miR-126 in normal epidermis was not expected with the LNA™ probe. With respect to miR-205 similar results were obtained using both ISH methods.

The results with the miR-21 COP ISH in the PC3 and LNCaP prostate cell lines were in line with the expression levels obtained using qRT-PCR reflecting their respective androgen independent and androgen sensitive derivation. This demarcation was also observed between the qualitative ISH and quantitative qRT-PCR. The staining in the Gleason stage 7 prostate biopsies confirmed an epithelial localisation for miR-21 in 74% of the 19 cases (Table 3) pointing to the utility of the use of COP probes for ISH.

The ISH results in the prostate tissues showed heterogeneity in demonstration of miR-21. This may reflect non-uniform expression due to underlying pathophysiology or it may point to the effect of formalin-based fixation. miRNA has been shown to be particularly resistant to the degrading effects of formalin on nucleic acids [33], but this does not imply that its effects will be uniform. This can be influenced by fixation time and the ease by which formalin can penetrate to all areas of a tissue to fix them.

From a practical view the LNA™ ISH protocol as described by Jørgensen, et al [9] provides results in an extended 'working' day while the COP ISH method is considerably longer. This difference should not be of consequence in a research environment, but could be a consideration in a diagnostic setting. An advantage of COP ISH is that it frees investigators from the need to purchase proprietary based LNA™ probes. COPs can also be acquired at reduced price and in quantities that can support their use over many more ISH runs.

This work represents an advance in biomedical science because it demonstrates that COPs can be used for ISH on FFPE samples with an expectation that they will produce equivalent results to of LNA™ probes for the demonstration of miRNAs.

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Summary table

What is known about this subject?

The expression levels of miRNAs can be quantitatively determined using molecular methods such as qRT-PCR.

In heterogenous samples the cellular localisation of differentially expressed miRNAs should be established using *in situ* hybridisation (ISH).

Using locked nucleic acid (LNA™) probes in ISH can reliably demonstrate miRNAs in formalin fixed paraffin embedded (FFPE) preparations.

What this paper adds?

ISH using conventional oligonucleotide probes (COP) is described providing equivalent results to the use of LNA™ in FFPE samples.

qRT-PCR undertaken on prostate cancer cell lines and COP ISH gave equivalent results.

The use of COPs for ISH provides economic savings in procurement while allowing for an increase in scale of use in experimental procedures.

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Table 1: Conventional oligonucleotide sequences

miRNA	**Sequence**	**GC content (%)**	**Melting temperature (°C)**
miR-21	5’- /5DigN/TCA ACA TCA GTC TGA TAA GCT A/3Dig_N/ -3’	36.4	39.1
miR-126	5’- /5DigN/ GCA TTA TTA CTC ACG GTA CGA /3Dig_N/ -3’	42.8	52.1
miR-205	5’- /5DigN/ AGA CTC CGG TGG AAT GAA GGA /3Dig_N/ -3’	52.3	57.8
Scrambled	5’- /5DigN/ GTG TAA CAC GTC TAT ACG CCC A/3Dig_N/ -3’	50	45.8

Legend

DigN = Digoxigenin NHS ester

Table 2: ***In situ*** **hybridisation methodologies**

	Fluorescein Poly d (T) probe	Digoxigenin labelled conventional oligonucleotide probes	Digoxigenin locked nucleic acid probes (Exiqon Instruction Manual v2.0)
Pre-treatment	Rehydrate sections, cover with Proteinase K and incubate for 30 mins at 37 °C, wash in DEPC water.		
Hybridisation	Dehydrate and air dry sections, add probe, incubate for 2 h at 37 °C, immerse in TBS.	Incubate with pre-hybridisation solution for 1 h at 37 °C. Add equal volume of hybridisation solution, incubate overnight at 37 °C. Immerse in TBS.	Dehydrate and air dry sections. Denature probes, hybridise for 1 h at 55 °C. Wash in 5 X SSC reducing to 0.2 X SSC at 55 °C then 0.2 X SSC at ambient temperature. Immerse in TBS.
Detection	Add TBSBT blocking solution, incubate for 10 mins. Tip off, add anti-FITC antibody conjugated to	As for poly d (T) probe except for use of anti-Digoxigenin	As for Digoxigenin labelled conventional probes except

	alkaline phosphatase (MB-2100, Vector Laboratories) diluted 1:100 in TBSBT and incubate for 30 mins. Rinse in TBS, wash in alkaline phosphatase substrate buffer. Cover sections with alkaline phosphatase substrate solution, incubate in dark for 1-2 h. Wash in water and mount using glycerol / water (3 / 1 VV).	antibody conjugated to alkaline phosphatase (11093274910, Roche) diluted 1:600 in TBSBT with overnight incubation in the alkaline phosphatase substrate.	incubation in alkaline phosphatase substrate for 1-2 h.

Legend

DEPC = Diethylpyrocarbonate treated water

TBS: 50 mM Tris-HCl, 150 mM sodium chloride, pH7.6

Pre-hybridisation solution: 600 mM sodium chloride,1 x PE buffer without SDS and bovine serum albumin, 10% dextran sulphate, 15µg / ml salmon sperm DNA, 30% formamide

Hybridisation solution: As for pre-hybridisation solution with addition of Digoxigenin labelled conventional oligonucleotide

TBSBT: TBS plus 0.1% Tween 20 and 3% bovine serum albumin

Alkaline phosphatase substrate buffer: 100mM TRIS, 50mM magnesium chloride hexahydrate, 100mM sodium chloride, pH 9.0.

Substrate solution: Ready to use Nitroblue tetrazolium / 5-Bromo-4-chloro-3-indolyl phosphate substrate solution (Sigma B1911-100ML) containing 1 mM levamisole and 0.2 -0.5μm pore size filtered before use.

SSC: Standard Saline Citrate buffer, pH 7.0.

Table 3 Prostate carcinoma ISH of 19 cases for miR-21 using conventional oligonucleotide probes

miR-21 staining patterns	**Cases**
Moderate to strong staining of some glands	2
Moderate staining of some glands	3
Weak to moderate staining of some glands	1
Weak staining of majority of glands	4
Weak staining of few glands	4
No specific staining	3
Unstained	2

Figure 1: Demonstration of miRNA by conventional oligonucleotide (COP) and locked nucleic acid (LNA) probes.

Legends

a) CRC, miR-126, COP probe, (x100 magnification). b) SCC of lung miR-126, COP probe, (x400 magnification). Note: strong staining of endothelium of capillaries and blood vessels.

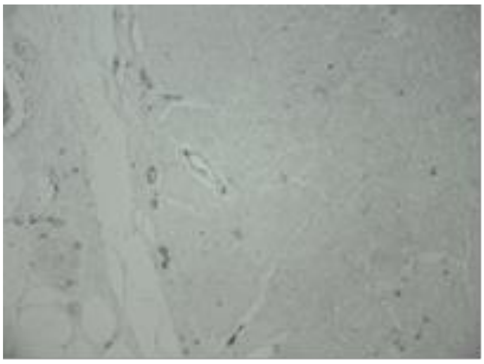
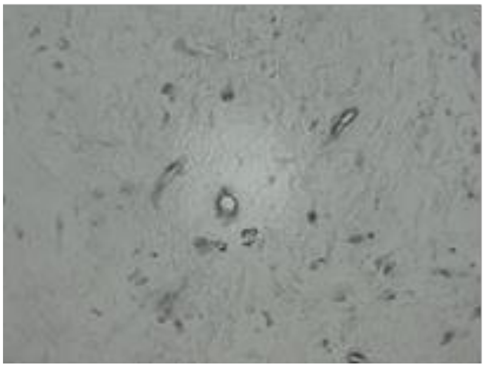
c) CRC, miR-205, COP probe, (x 100 magnification). Note: strong staining of normal epithelial glands (left) and moderate staining of adenocarcinoma (right). d) SCC of lung, miR-205, COP probe, (x 400 magnification). Note: strong staining of tumour islands.

IBC e) miR-126 COP probe, (x100 magnification). Note: Moderate staining of tumour islands (right) together with strong staining of blood vessels (arrows). f) miR-126 LNA™ probe, (x100 magnification). Note: strong staining of tumour islands (right).

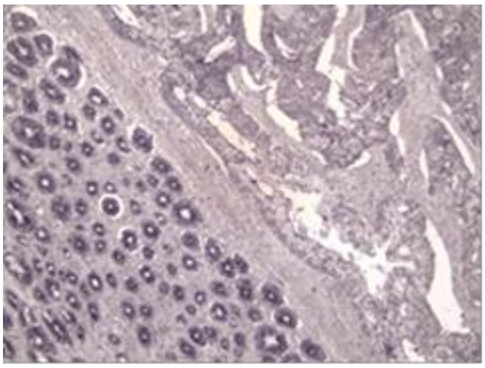
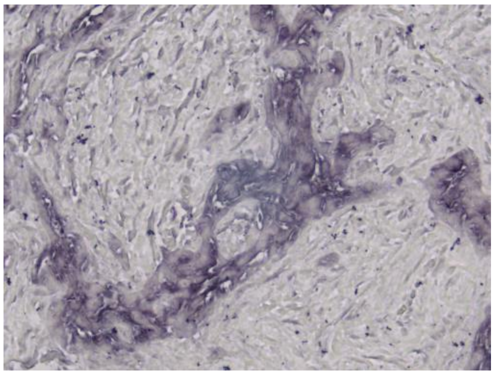
SCC of lung: g) miR-205 COP probe, (x100 magnification). h) miR-205 LNA™ probe, (x100. magnification) Note: strong staining of tumour islands with both probes

Prostate carcinoma miR-21COP ISH, (x400 magnification): i) PC3 cell block section. Note: strong staining of several cells j) LNCaP cell block section. Note absence of specific staining. k) Gleason stage 7 tissue section. Note: moderate to strong staining of cells within tumour gland (arrow).

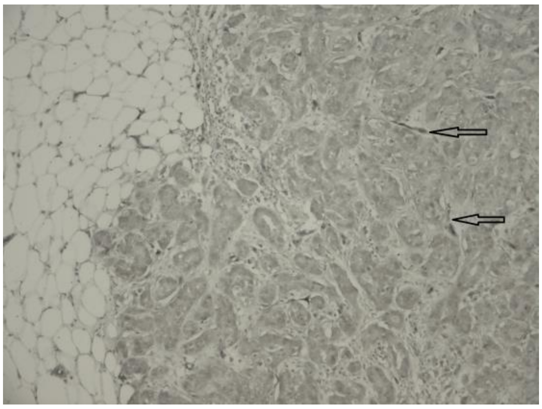
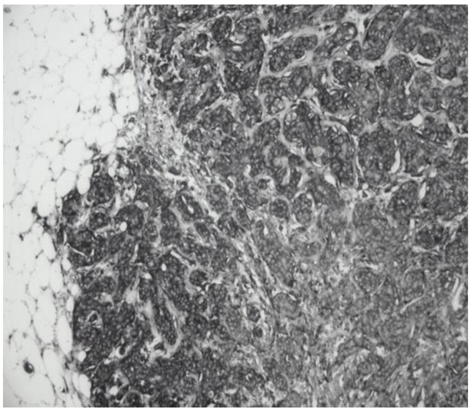
Figure 1: Demonstration of miRNA by conventional oligonucleotide (COP) and locked nucleic acid (LNA) probes.

 a)  b)

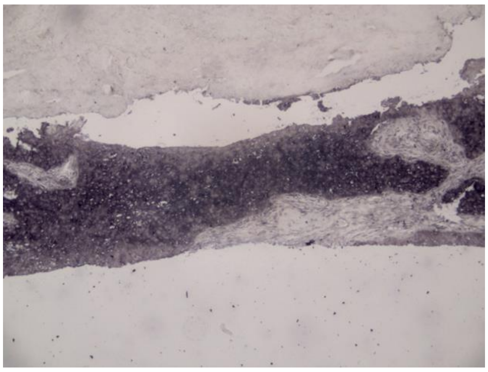
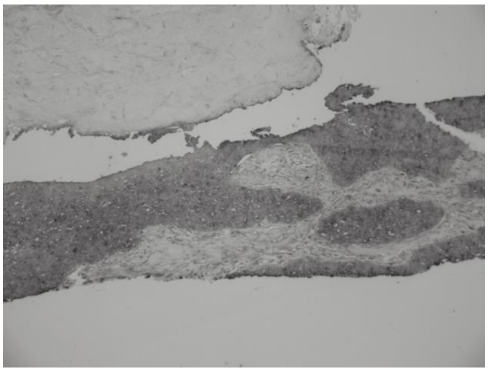
a) CRC, miR-126, COP probe, (x100 magnification). b) SCC of lung miR-126, COP probe, (x400 magnification). Note: strong staining of endothelium of capillaries and blood vessels.

 c)  d)

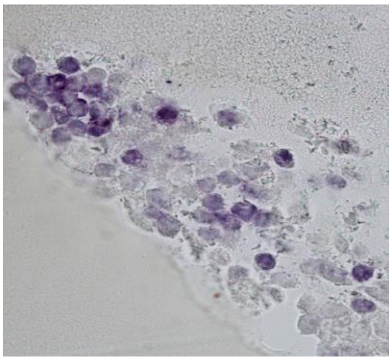
c) CRC, miR-205, COP probe, (x 100 magnification). Note: strong staining of normal epithelial glands (left) and moderate staining of adenocarcinoma (right). d) SCC of lung, miR-205, COP probe, (x 400 magnification). Note: strong staining of tumour islands.

 e)  f)

IBC e) miR-126 COP probe, (x100 magnification). Note: Moderate staining of tumour islands (right) together with strong staining of blood vessels (arrows). f) miR-126 LNA™ probe, (x100 magnification). Note: strong staining of tumour islands (right).

 g)  h)

SCC of lung: g) miR-205 COP probe, (x100 magnification). h) miR-205 LNA™ probe, (x100. magnification) Note: strong staining of tumour islands with both probes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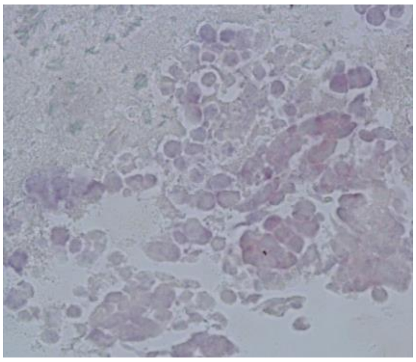
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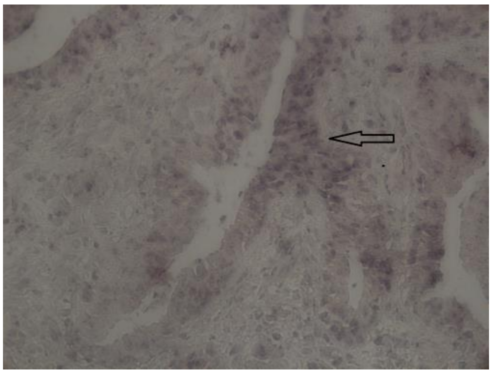

k)

Prostate carcinoma miR-21COP ISH, (x400 magnification): i) PC3 cell block section. Note: strong staining of several cells j) LNCaP cell block section. Note absence of specific staining. k) Gleason stage 7 tissue section. Note: moderate to strong staining of cells within tumour gland (arrow).

Table 1: Conventional oligonucleotide sequences

miRNA	Sequence	GC content (%)	Melting temperature (°C)
miR-21	5’- /5DigN/TCA ACA TCA GTC TGA TAA GCT A/3Dig_N/ -3’	36.4	39.1
miR-126	5’- /5DigN/ GCA TTA TTA CTC ACG GTA CGA /3Dig_N/ -3’	42.8	52.1
miR-205	5’- /5DigN/ AGA CTC CGG TGG AAT GAA GGA /3Dig_N/ -3’	52.3	57.8
Scrambled	5’- /5DigN/ GTG TAA CAC GTC TAT ACG CCC A/3Dig_N/ -3’	50	45.8

Legend

DigN = Digoxigenin NHS ester

Table 2: *In situ* hybridisation methodologies

	Fluorescein Poly d (T) probe	Digoxigenin labelled conventional oligonucleotide probes	Digoxigenin locked nucleic acid probes (Exiqon Instruction Manual v2.0)
Pre-treatment	Rehydrate sections, cover with Proteinase K and incubate for 30 mins at 37 °C, wash in DEPC water.		
Hybridisation	Dehydrate and air dry sections, add probe, incubate for 2 h at 37 °C, immerse in TBS.	Incubate with pre-hybridisation solution for 1 h at 37 °C. Add equal volume of hybridisation solution, incubate overnight at 37 °C. Immerse in TBS.	Dehydrate and air dry sections. Denature probes, hybridise for 1 h at 55 °C. Wash in 5 X SSC reducing to 0.2 X SSC at 55 °C then 0.2 X SSC at ambient temperature. Immerse in TBS.
Detection	Add TBSBT blocking solution, incubate for 10 mins. Tip off, add anti-FITC antibody conjugated to	As for poly d (T) probe except for use of anti-Digoxigenin	As for Digoxigenin labelled conventional probes except

	alkaline phosphatase (MB-2100, Vector Laboratories) diluted 1:100 in TBSBT and incubate for 30 mins. Rinse in TBS, wash in alkaline phosphatase substrate buffer. Cover sections with alkaline phosphatase substrate solution, incubate in dark for 1-2 h. Wash in water and mount using glycerol / water (3 / 1 VV).	antibody conjugated to alkaline phosphatase (11093274910, Roche) diluted 1:600 in TBSBT with overnight incubation in the alkaline phosphatase substrate.	incubation in alkaline phosphatase substrate for 1-2 h.

Legend

DEPC = Diethylpyrocarbonate treated water

TBS: 50 mM Tris-HCl, 150 mM sodium chloride, pH7.6

Pre-hybridisation solution: 600 mM sodium chloride,1 x PE buffer without SDS and bovine serum albumin, 10% dextran sulphate, 15μg / ml salmon sperm DNA, 30% formamide

Hybridisation solution: As for pre-hybridisation solution with addition of Digoxigenin labelled conventional oligonucleotide

TBSBT: TBS plus 0.1% Tween 20 and 3% bovine serum albumin

Alkaline phosphatase substrate buffer: 100mM TRIS, 50mM magnesium chloride hexahydrate, 100mM sodium chloride, pH 9.0.

Substrate solution: Ready to use Nitroblue tetrazolium / 5-Bromo-4-chloro-3-indolyl phosphate substrate solution (Sigma B1911-100ML) containing 1 mM levamisole and 0.2 -0.5μm pore size filtered before use.

SSC: Standard Saline Citrate buffer, pH 7.0.

Table 3 Prostate carcinoma ISH of 19 cases for miR-21 using conventional oligonucleotide probes

miR-21 staining patterns	**Cases**
Moderate to strong staining of some glands	2
Moderate staining of some glands	3
Weak to moderate staining of some glands	1
Weak staining of majority of glands	4
Weak staining of few glands	4
No specific staining	3
Unstained	2

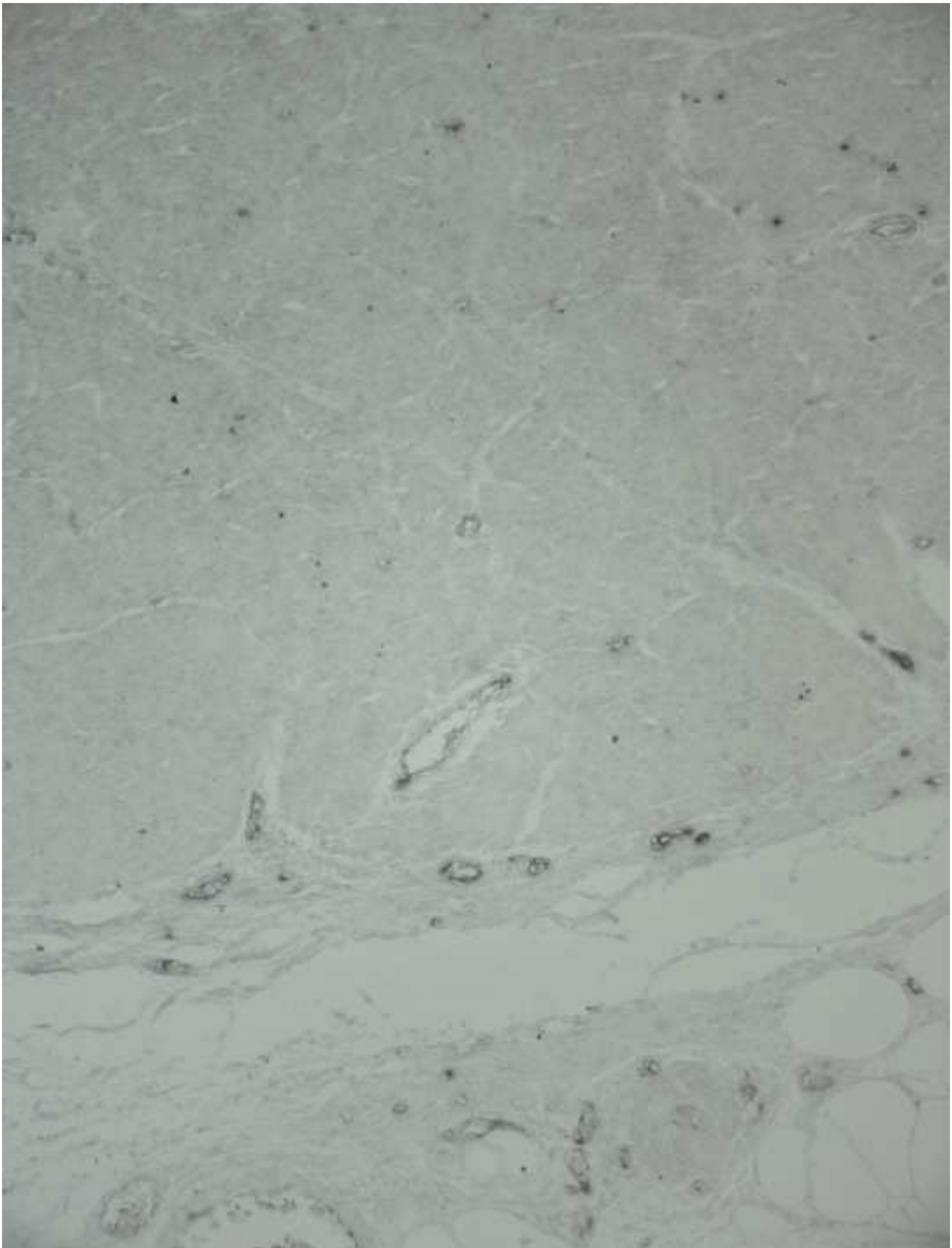
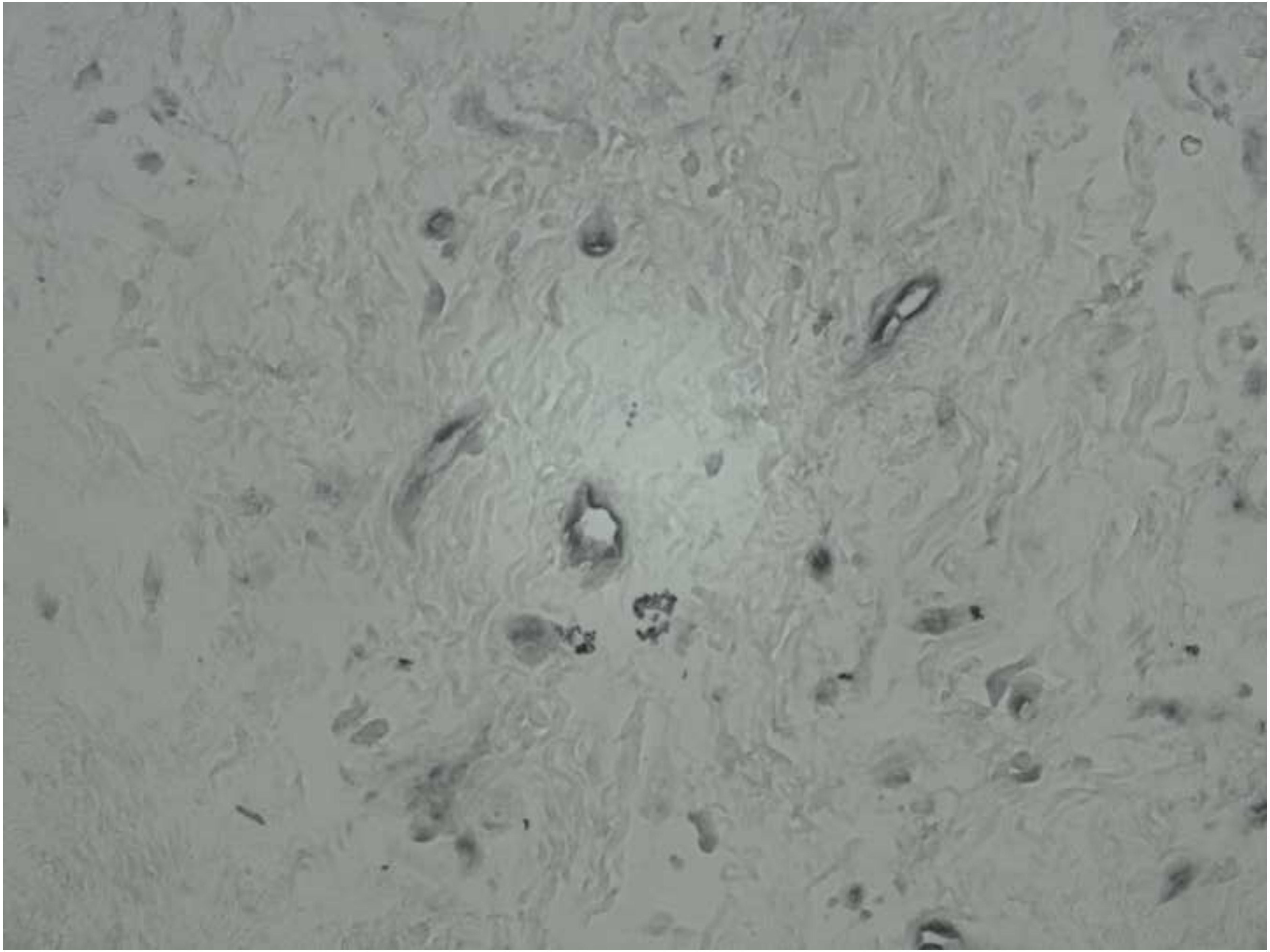


figure 1

figure 1



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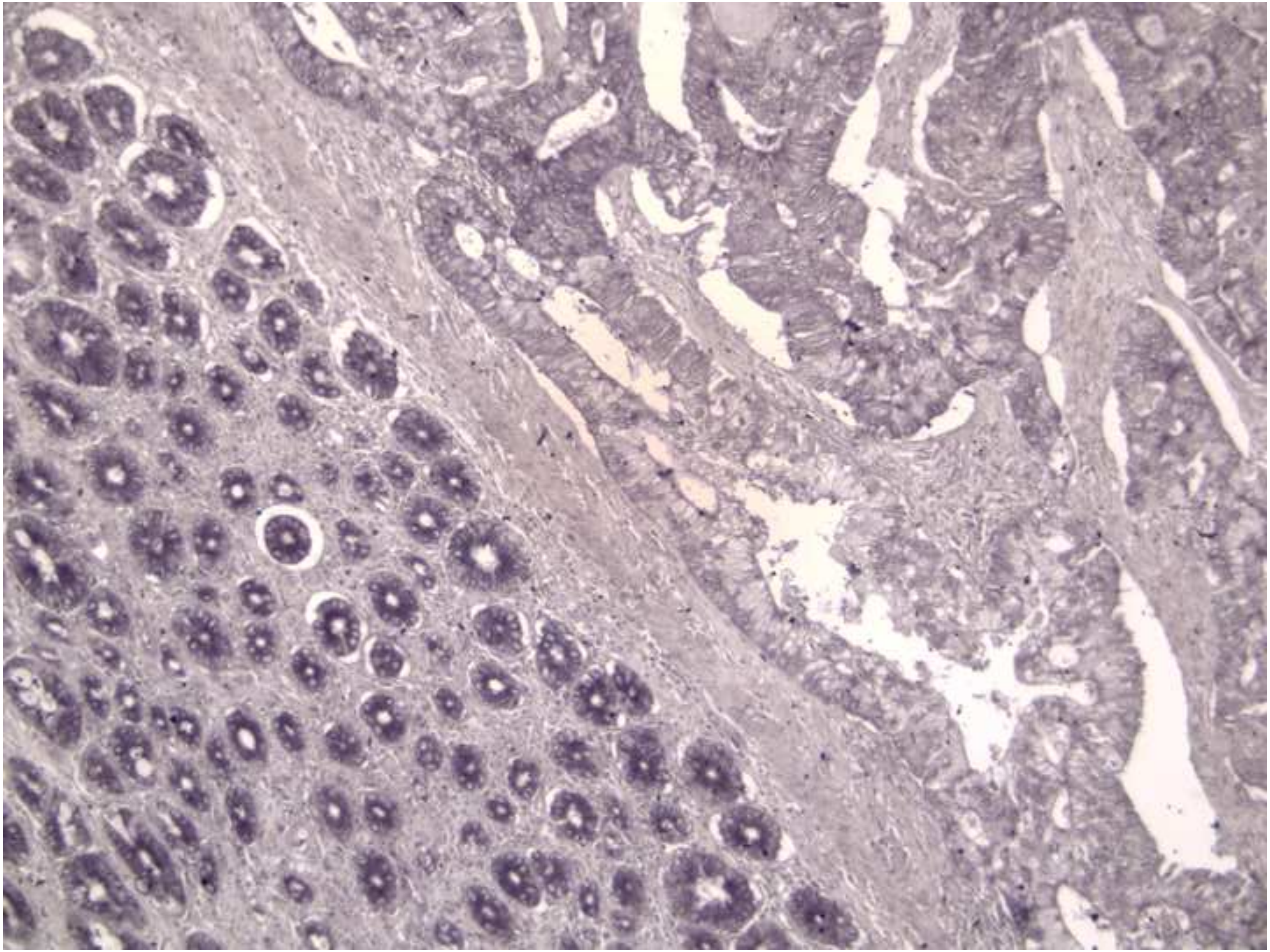

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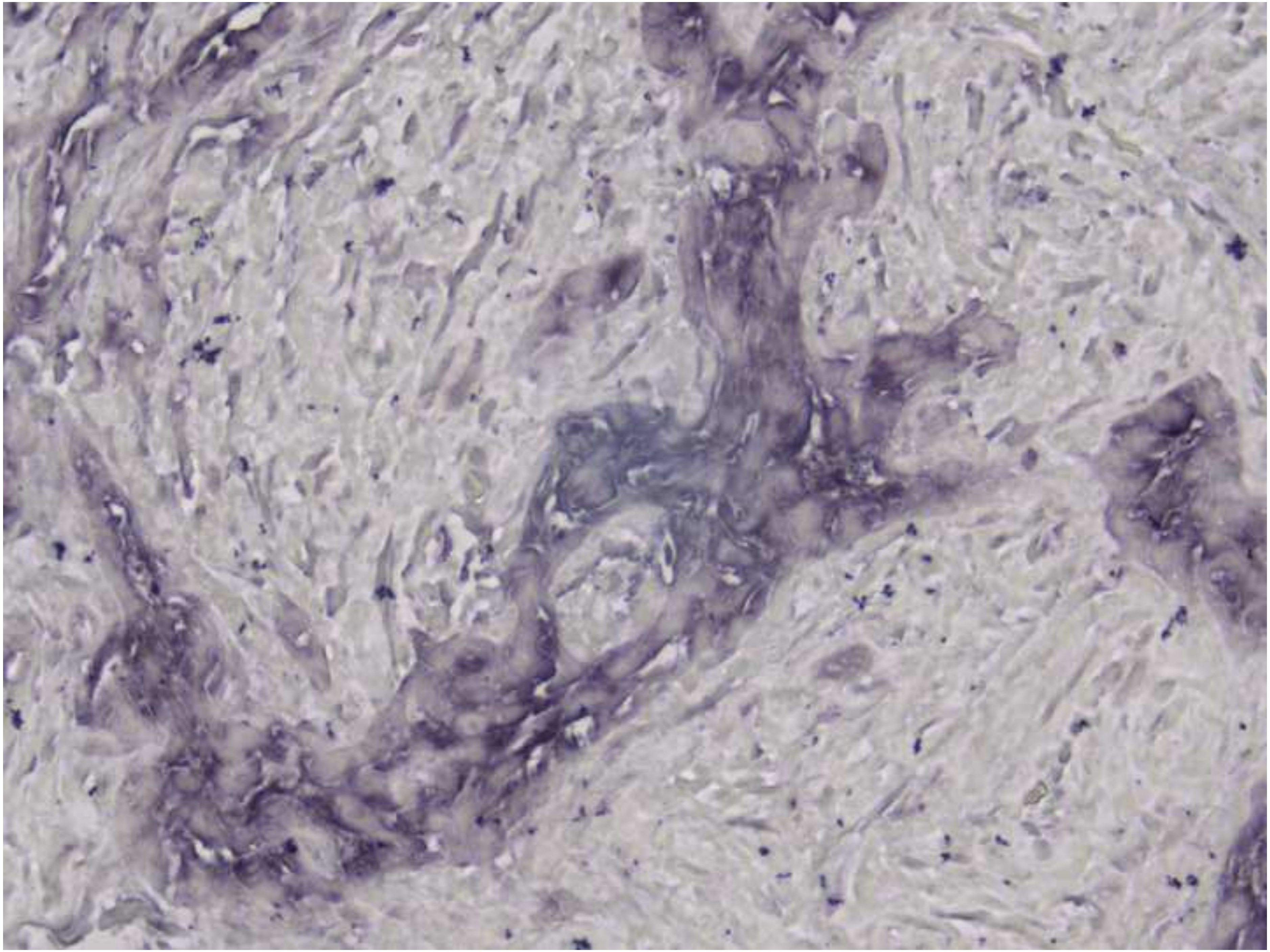

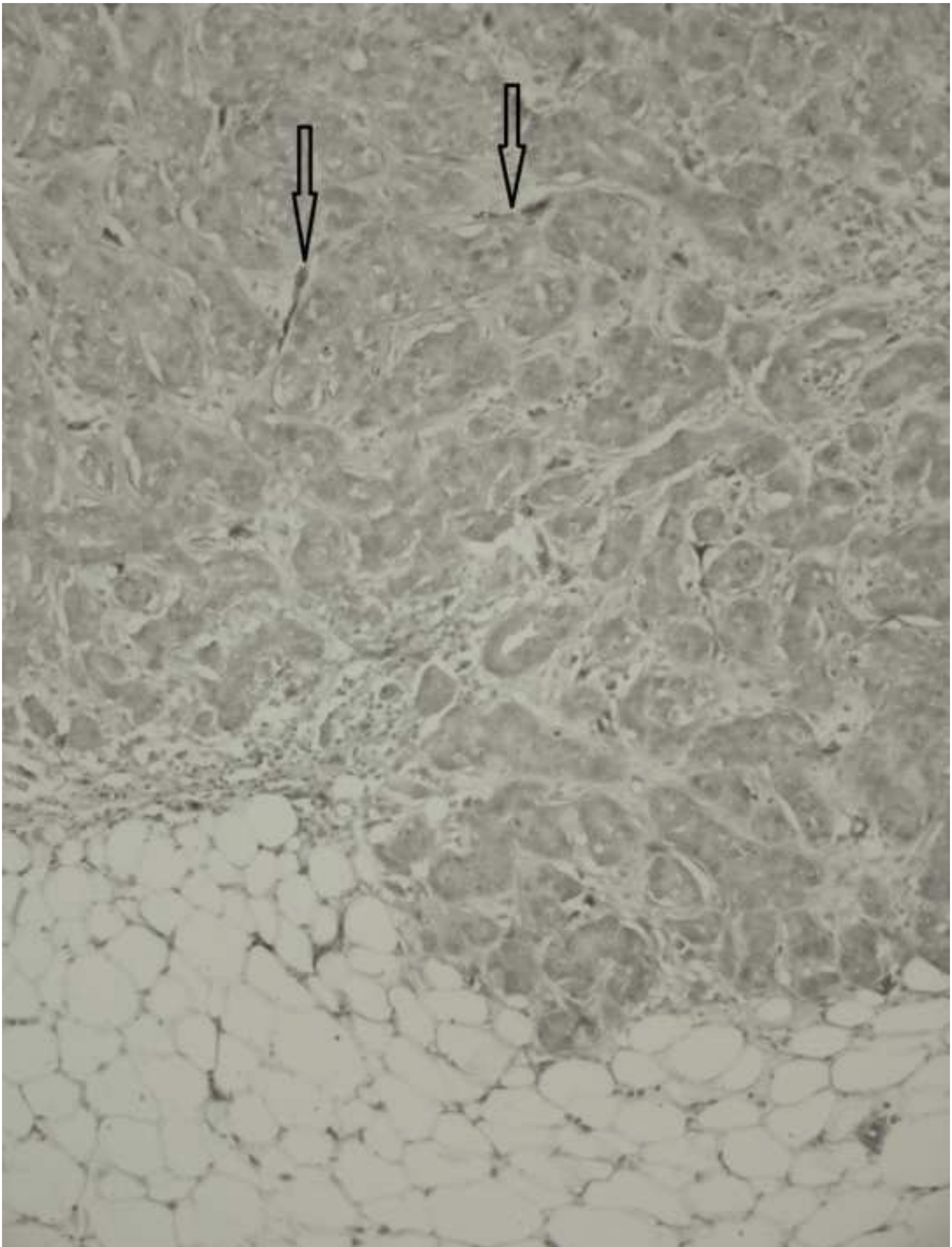

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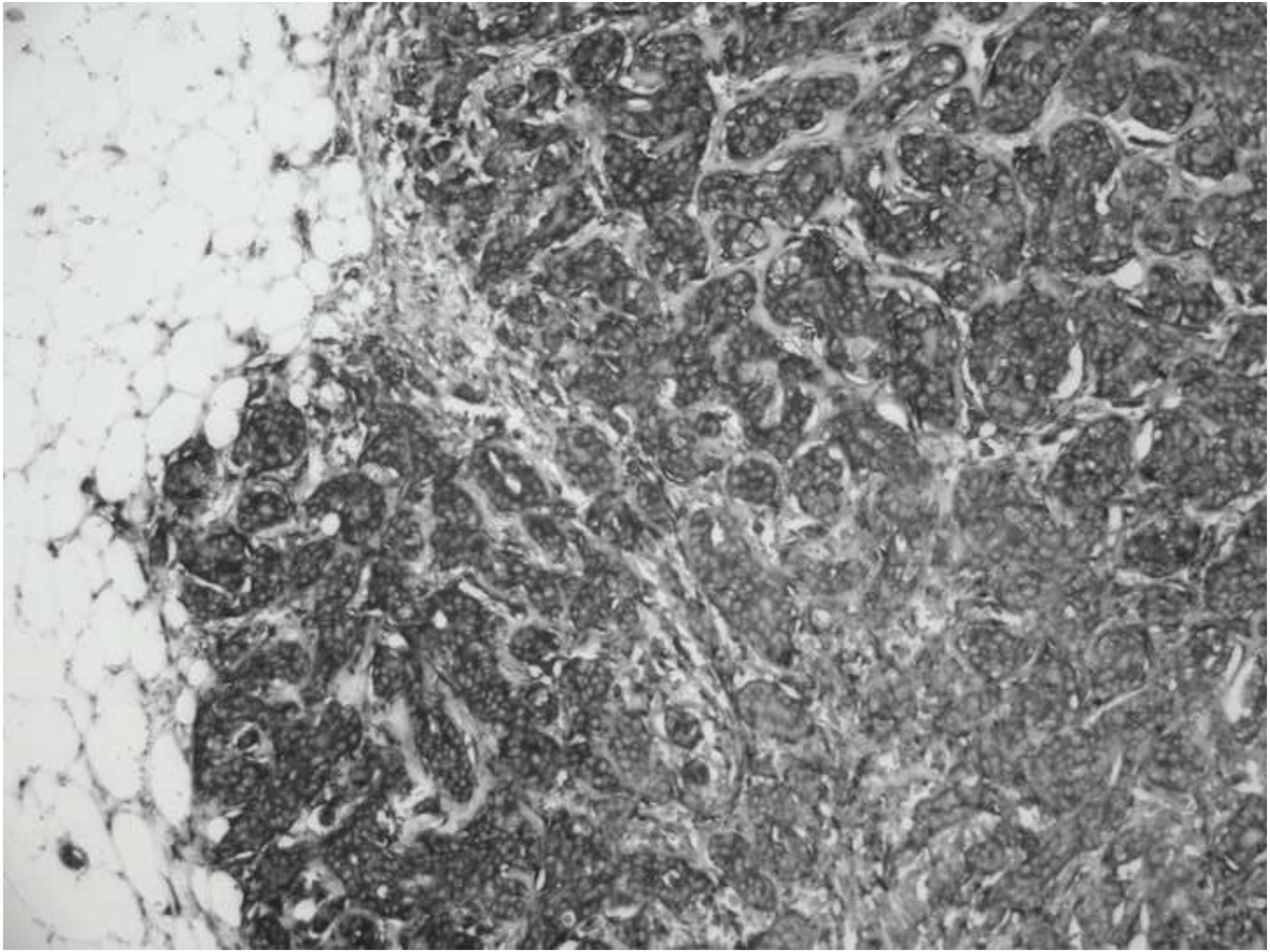

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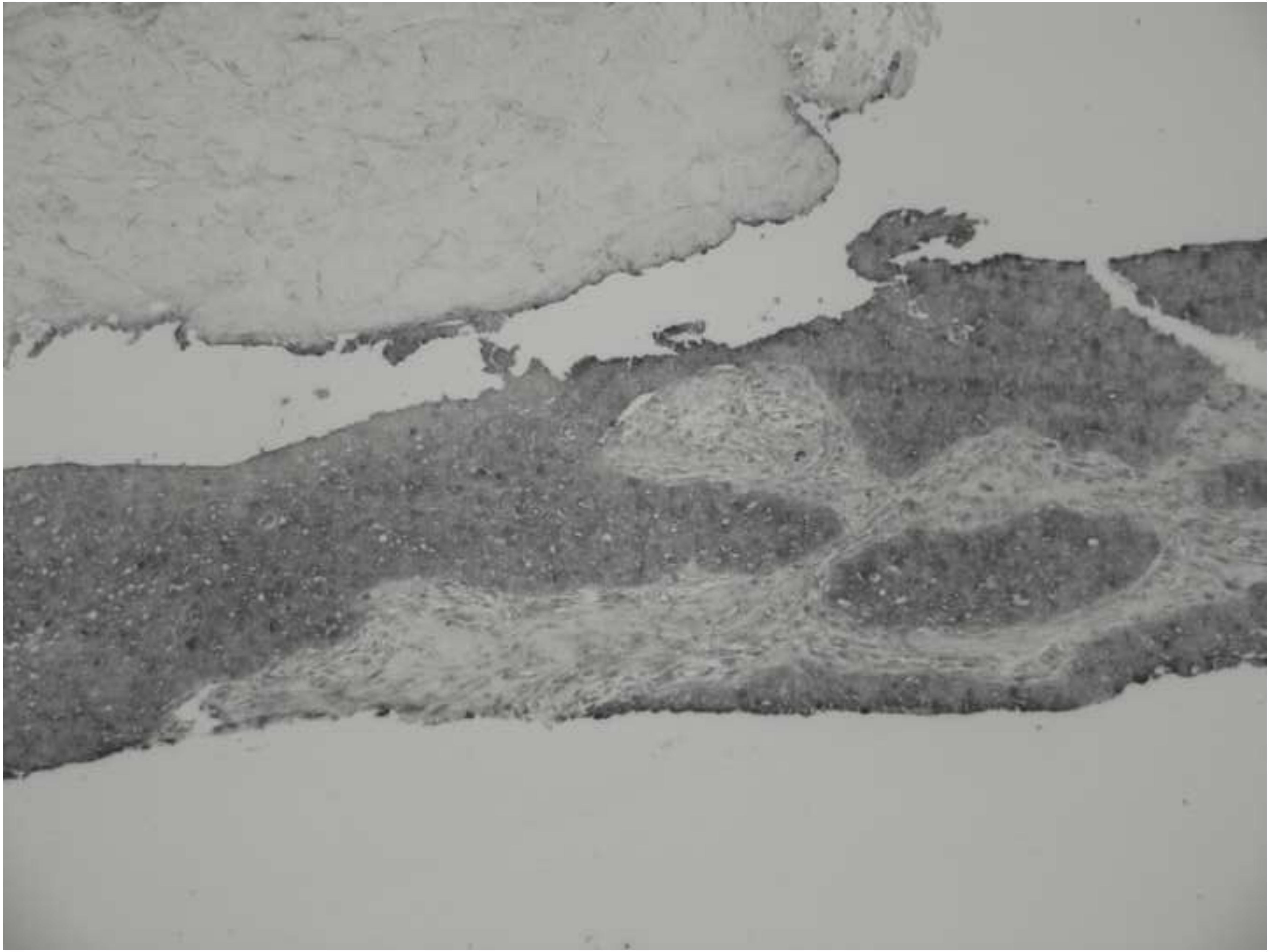

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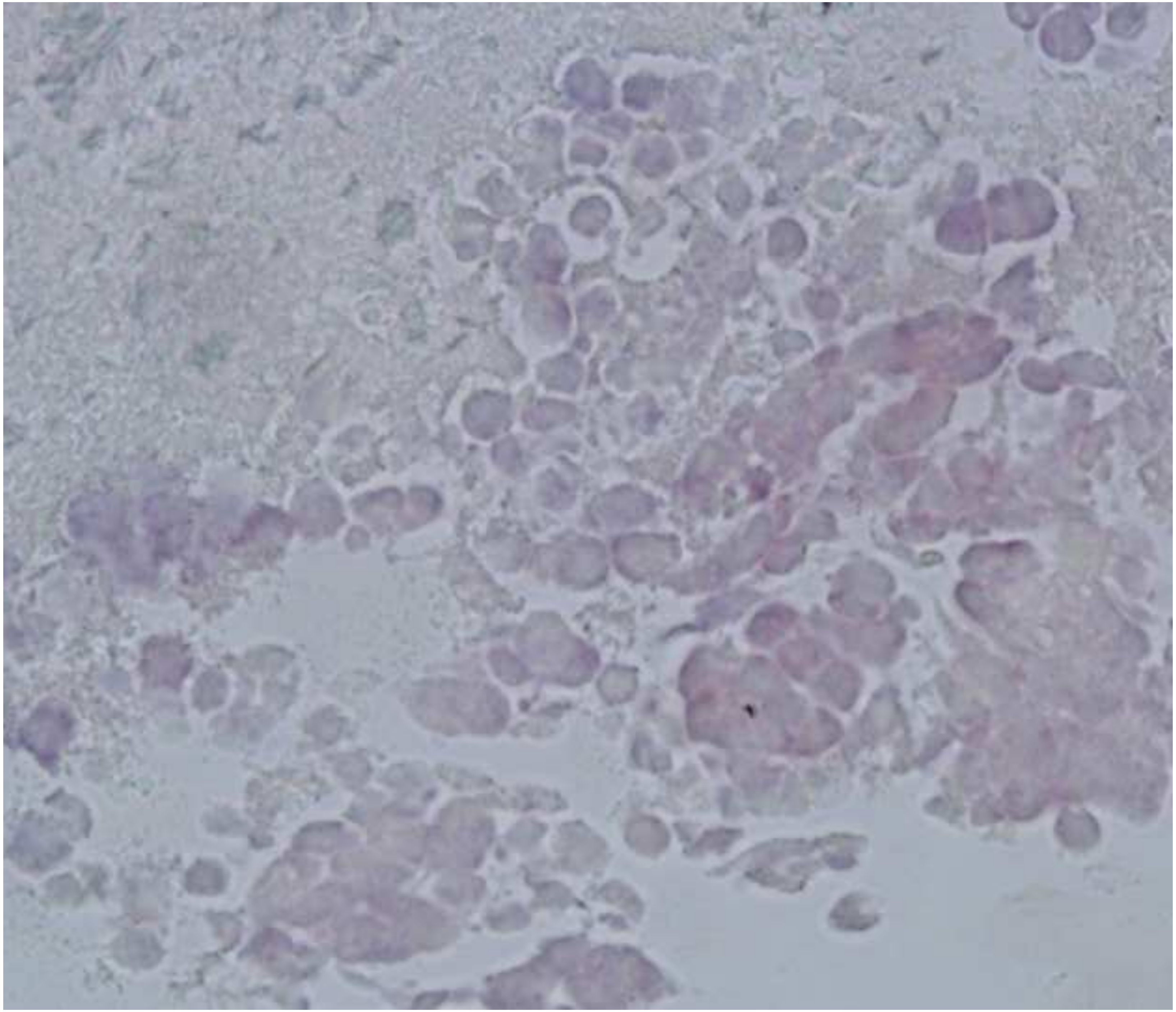

figure 1