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Impact of polymorphisms in the 5’-UTR of glutathione S-transferase P1 on proliferative activity in canine mammary tumours

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Abstract

Background

Mammary tumours are a prevalent malignancy in female dogs. Successful treatment of this pathology requires early diagnosis using molecular genetic methods. Single-nucleotide polymorphisms (SNPs) in genes such as *GSTP1* may serve as potential diagnostic markers. This study aimed to identify associations between polymorphisms in the 5'-untranslated region and the proximal promoter of *GSTP1* in dogs and mammary tumour occurrence, and to examine their relationship with the Ki-67 proliferation index.

Methodology

This study included 50 female dogs diagnosed with mammary tumours. Blood samples were collected in EDTA tubes and refrigerated until DNA extraction. Surgically excised tumour tissues were examined histologically. DNA was extracted from blood, amplified by PCR, and the resulting PCR products were purified and sequenced by Sanger method. Immunohistochemistry was used to evaluate Ki-67 expression.

Results

A polymorphism was identified in the proximal promoter region, and twelve polymorphisms, including nine SNPs and three deletions, were found in the 5'-UTR of *GSTP1*. These polymorphisms were grouped into seven haplotypes, and the combined haplotype group showed a statistically significant difference compared with healthy dogs (OR 21.176, 95% CI 2.548-175.974, P<0.001), although individual haplotypes were not statistically significant. Immunohistochemistry for Ki-67 in 18 epithelial mammary tumour samples revealed the lowest proliferation index in Hap0 (15.95% ± 5.11), which was significantly lower than the mean proliferation index in the other affected dogs (24.09% ± 3.49).

Conclusions

This study confirms significant associations between polymorphisms of the 5'-UTR of *GSTP1*and canine mammary tumours, particularly within haplotype groups. The lower Ki-67 proliferation index in Hap0 suggests potential prognostic relevance for these polymorphisms.

Introduction

Mammary tumours are among the most common tumours in dogs [1, 2]. Similarly, breast tumours have a high incidence rate among women worldwide [3]. The search for biomarkers that enable early diagnosis of this pathology remains ongoing [4]. Single-nucleotide polymorphisms (SNPs) in genes could serve this role. One such gene is *GSTP1*, which encodes an enzyme that catalyses the conjugation of reduced glutathione to electrophilic substrates, including carcinogens [5]. There are two directions in studying *GSTP1*: the association of gene polymorphisms with tumour occurrence and the high enzymatic activity of *GSTP1* as a mechanism of drug resistance in cancer cells [6].

Board P.G. et al. [7] first described two amino acid substitutions in *GSTP1* – Ile105Val and Ala114Val, which led to decreased enzymatic activity [8]. The Ile105Val substitution of *GSTP1* is the result of the c.313 A > G polymorphism of the *GSTP1*, which has been described in numerous studies. In humans, associations with breast cancer [9], stomach cancer [10], and prostate and bladder cancers [11] were investigated. Ge et al. [12] showed that women with the c.313 A > G polymorphism have a higher risk of breast cancer, especially histological grade III.

Identifying new biomarkers of mammary tumours in dogs is also important for early diagnosis and successful treatment. Additionally, new knowledge about this pathology in canines may be useful for women's breast cancer research.

In dogs, elevated *GST* activity [13] and *GSTP* expression [14, 15] have been observed in mammary tumour tissues. Such changes could result from polymorphisms of *GSTP1.* For now, these polymorphisms cannot be considered valid tumour markers due to insufficient knowledge. As noted by Sacco [16], the canine *GSTP1* locus shows significant functional genetic variation that may influence enzyme expression and cancer susceptibility in dogs.

Ki-67 is a nuclear protein associated with cellular proliferation, and its expression has been linked to malignancy and reduced survival rates in dogs with mammary tumours, highlighting its importance as a prognostic marker [17, 18].

Potential polymorphisms in exons, introns, and the 5' untranslated region (5'-UTR) of *GSTP1* in dogs with mammary tumours were examined in previous work by the authors of the current study [19].

The aim of the current study was to investigate the entire 5'-UTR region and the proximal part of promoter *GSTP1* among a larger number of experimental animals. Moreover, the authors aimed to identify associations between polymorphisms and the occurrence of mammary tumours and the Ki-67 proliferation index.

Taking this into consideration, polymorphisms and the proliferation index were compared, with the assumption that SNPs may also serve as prognostic markers.

Materials and methods

2.1 Sample collection

For the investigation, 50 client-owned female dogs with mammary tumours in Lviv (Ukraine) and Lublin (Poland) were included from August 2015 to February 2025. The control group consisted of 15 healthy female dogs without a prior cancer diagnosis. Blood samples from dogs were collected in EDTA-containing tubes and stored at 4°C until DNA extraction. The tissues from surgically removed mammary tumours were fixed in 10% neutral formalin. Formalin-fixed samples were embedded in paraffin, sectioned and stained with haematoxylin and eosin (HE) for histological examination. Classification of tumours was carried out according to Goldschmidt et al. [20]. The cell proliferation activity was determined immunohistochemically (IHC) with the use of primary mouse monoclonal antibody anti-Ki-67 (clone MIB-1, Dako, Glostrup,

Denmark) at a dilution of 1:500. The technique employed a system for antigen-antibody complexes based on secondary biotin-linked antibodies directed against mouse primary monoclonal antibodies DAKO-REAL, En-Vision HRP (Dako, Glostrup, Denmark). The enzyme used to label the reaction site was horseradish peroxidase conjugated with streptavidin. The chromogen was tetrahydrochloride-3,3'- diaminobenzidine (DAB, SK-4100, Vector Laboratories, Peterborough, UK). The IHC-stained slides were scanned at 40x magnification with a resolution of 0.23 μm/pixel using a digital slide scanner (Pannoramic MIDI, 3DHISTECH, Budapest, Hungary). The digital images were analysed for Ki-67 protein expression using the QuantCenter software v. 2.4 (3DHISTECH, Budapest, Hungary). The Ki-67 index was calculated as the percentage of positive cells in 1000 tumour cells.

2.2 DNA extraction and genotyping

DNA was extracted from blood using the Blood Mini Kit (A&A Biotechnology, Gdańsk, Poland). The quality and purity of the DNA were detected spectrophotometrically and by agarose gel electrophoresis. Primers CAGCAGGGTTCGGGTCCCGCAG (forward) / TCCAGCCGGGAGCATTCCGAGG (reverse) were used for amplification, and their properties were calculated by OligoCalc [21]. DNA was amplified by PCR with Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific Inc., Waltham, Massachusetts) according to the manufacturer's specifications. The reaction mixture contained 0.5 μM of each primer, 200 μM dNTPs, HF buffer, 5% DMSO, and 50 ng of chromosomal DNA as the template in a total reaction volume of 50 μL. Conditions of amplification: initial denaturation at 98°C for 3 min, followed by 35 cycles of denaturation at 98°C for 15 s, annealing-elongation at 72°C for 30 s, and final elongation after these cycles at 72°C for 10 min (Biometra T-Personal Combi, Analytik Jena GmbH + Co. KG, Germany). The PCR products were separated on a 0.8% agarose gel (ethidium bromide staining) and purified using the ZymoClean Gel DNA Recovery Kit (Zymo Research, Irvine, California). Purified PCR products were sequenced by Sanger sequencing method, and polymorphisms were screened using the MultAlin software [22]. To identify polymorphisms, the sequences were manually analysed and visually confirmed by comparison with the canine *GSTP1* sequence assembly ROS_Cfam_1.0 (chromosome 18: c50830507-50827487) from the National Center for Biotechnology Information. *In silico* analyses of predicted splicing effects were evaluated using the modelling programmes ESEfinder [23, 24] and Human Splicing Finder [25].

2.3 Statistical analysis

Genotypic polymorphisms were examined for deviation from the Hardy–Weinberg equilibrium using the chi-square test with one degree of freedom. Data were analysed using IBM SPSS Statistics version 31.0.0, employing descriptive statistics. The association between *GSTP1* polymorphisms and the occurrence of mammary tumours was assessed using logistic regression to estimate odds ratios (OR), 95% confidence interval (CI), and two-sided Fisher's exact test. A probability of $P<.05$ was considered statistically significant.

Results

The studied population consisted of 50 dogs with mammary tumours (Table 1) with an average age of 8.68 ± 0.25 years (range 4.5–12.5). A control group consisted of 15 healthy German Shepherd female dogs,

with an average age of 7.61 ± 0.47 years (range 5.7–11.5), comparable to that of the sick animals. After the end of the study period, no mammary tumours were diagnosed in any healthy animals.

The histopathological diagnoses of the tumours are presented in Table 1. All tumours examined were malignant; 92% were epithelial, and 8% were mesenchymal. The grade distribution was as follows: I – 36.7%, II – 55.1%, and III – 8.2%. According to clinical observations, the first stage was observed in 28% of sick animals, stage II in 10%, stage III in 49%, and stage IV in 13%.

Table 1
Characteristics of the studied population of dogs with mammary tumours.

Breed	No. samples	Histopathological diagnosis	No. samples
Overall	50	Epithelial tumours	46
		Mesenchymal tumours	4
German Shepherd	22	Tubulopapillary carcinoma	9
		Ductal carcinoma	3
		Cystic-papillary carcinoma	1
		Mucinous carcinoma	1
		Solid carcinoma	2
		Carcinoma mixed type	3
		Fibrosarcoma	2
		Tubular carcinoma	1
Basenji	1	Tubular carcinoma	1
Bernese Mountain Dog	1	Ductal carcinoma	1
Cane Corso	2	Carcinoma cribriform	2
Chihuahua	2	Ductal carcinoma	1
		Chondrosarcoma	1
Dachshund	1	Comedocarcinoma	1
English Cocker Spaniel	3	Carcinoma mixed type	1
		Carcinoma complex type	1
		Tubular carcinoma	1
German Hunting Terrier	1	Mucinous carcinoma	1
Jack Russell Terrier	1	Carcinoma cribriform	1
Pomeranian Shpitz	1	Tubulopapillary carcinoma	1
Rottweiler	1	Osteosarcoma	1
Russo-European Laika	1	Carcinoma cribriform	1
Siberian Husky	1	Carcinoma mixed type	1
Standard Schnauzer	1	Ductal carcinoma	1

Breed	No. samples	Histopathological diagnosis	No. samples
Yorkshire Terrier	3	Tubulopapillary carcinoma	1
		Ductal carcinoma	1
		Carcinoma mixed type	1
Zwergpinscher	1	Tubulopapillary carcinoma	1
Mixed breed	7	Lipid-rich carcinoma	1
		Tubulopapillary carcinoma	1
		Solid carcinoma	1
		Tubular carcinoma	1
		Squamous cell carcinoma	1
		Carcinoma mixed type (mesenchymal component)	1
		Ductal carcinoma	1

One polymorphism was found in the proximal region of the promoter, and 12 polymorphisms were identified in the 5'-UTR of *GSTP1*: 9 SNPs and 3 deletions (Table 2, Supplementary Table 1). All polymorphisms were found in sick animals. The healthy dogs had no polymorphisms, except for one, in which 5'-UTR c.-21 A to G and 5'-UTR c.-46_-44 delTGC were noted. The minor allele frequencies of the polymorphisms and their characteristics are presented in Table 2.

Table 2
Minor allele frequencies of *GSTP1* SNPs and deletions in dogs with mammary tumours (n = 50) and healthy dogs (n = 15).

Variant ID	Chromosome: bp (ROS_Cfam_1.0)	Nucleotide	Base pairs change	Minor allele frequencies	
				Mammary tumours	Healthy
rs1152388475	18:50830550	c.-127	G to C	0.04	0.00
rs1152388474	18:50830491	5'-UTR c.-68	C to T	0.12	0.00
rs3342361377	18:50830475	5'-UTR c.-52	C to T	0.12	0.00
rs3341757173	18:50830471	5'-UTR c.-48	G to A	0.12	0.00
rs22633673	18:50830469	5'-UTR c.-46	T to C	0.04	0.00
rs1152388497	18:50830467–50830469	5'-UTR c.-46_-44	deletion TGC	0.28	0.02
rs1152388496	18:50830466	5'-UTR c.-43	C to T	0.12	0.00
		5'-UTR c.-42	G to A	0.12	0.00
		5'-UTR c.-30	G to A	0.12	0.00
rs3341757161	18:50830452–50830469	5'-UTR c.-46_-29	deletion TGCCGCCGCCGCCGCCGC	0.14	0.00
rs1152388473	18:50830450	5'-UTR c.-27	G to A	0.04	0.00
rs5506102839	18:50830445–50830447	5'-UTR c.-24_-22	deletion GCC	0.12	0.00
rs1152388472	18:50830444	5'-UTR c.-21	A to G	0.18	0.02

The samples were grouped into 7 haplotypes based on the similarity of their polymorphisms (Table 3). Fourteen healthy animals and dogs with mammary tumours that had no polymorphisms were assigned to Hap0. In the studied population of 50 sick animals, 26 had one of the following haplotypes: Hap1, Hap2, Hap3, Hap4, Hap5, Hap6. The difference between this group and the group of healthy animals was statistically significant according to both confidence interval (OR 15.167, 95% CI 1.851-124.257) and a two-sided Fisher's exact test (P = 0.002).

The most common haplotypes were Hap1, Hap3, and Hap5. The statistical differences between haplotypes of sick and healthy animals are presented in Table 3.

Table 3
Comparison of *GSTP1* haplotypes in dogs with mammary tumours.

Haplotype	SNPs and deletions	Number of sick dogs	OR (95% CI)	P-value*	Transcriptional Factor Impact (Create/Abolish)
Hap 0		24	0.066 (0.008–0.540)	0.002	-
Hap 1	5'-UTR c.-21, A > G 5'-UTR c.-46-44, delTGC	7	2.279 (0.258–20.169)	0.669	ESE_SC35, PESE, ESE_ASFB, ESE_SRp40, EIE, PESS, Sironi_motif3, ESE_SRp55, RESCUE ESE.
Hap 2	5'-UTR c.-21, A > G 5'-UTR c.-46, T > C	2	0.625 (0.053–7.385)	1.000	ESE_SC35, PESE, ESE_9G8, ESE_ASFB, ESE_SRp40, EIE, PESS, Sironi_motif3, ESE_SRp55, ESE_ASF.
Hap 3	5'-UTR c.-24-22, delGCC 5'-UTR c.-30, G > A 5'-UTR c.-42, G > A 5'-UTR c.-43, C > T 5'-UTR c.-48, G > A 5'-UTR c.-52, C > T 5'-UTR c.-68, C > T	6	2.045 (0.227–18.398)	1.000	ESE_SC35, PESE, ESE_9G8, ESE_ASFB, ESE_SRp40, EIE, PESS, Sironi_motif3, ESE_SRp55, ESE_ASF, RESCUE, ESE, IIE.
Hap 4	5'-UTR c.-27, G > A	2	0.625 (0.053–7.385)	1.000	ESE_SC35, PESE, ESE_9G8, ESE_ASFB, ESE_SRp40, EIE, PESS, Sironi_motif3
Hap 5	5'-UTR c.-46-29, delTGCCGCCGCCGCCGCCGC	7	2.442 (0.277–21.519)	0.668	ESE_SC35, PESE, ESE_9G8, ESE_ASFB, ESE_SRp40, EIE, PESS, Sironi_motif3, ESE_SRp55, ESE_ASF, RESCUE, ESE.
Hap 6	c.-127, G > C	2	0.625 (0.053–7.385)	1.000	

*P<.05 are indicated in bold

Each haplotype possessed a unique polymorphism, with no polymorphisms shared across different haplotypes. There were two exceptions. The first is the SNP A-to-G in the 5'-UTR c.-21, which was found in 9 samples (Hap1, Hap2), but neither the confidence interval (OR 3.073, 95% CI 0.357–26.468) nor the two-sided Fisher's exact test (P = 0.431) showed a significant difference. The second is a deletion of TGC in the 5'-UTR c.-46-44. Fourteen dogs with the deletion were identified among the entire population of sick animals. The difference relative to the group of healthy dogs was also not statistically significant, based on the confidence interval (OR 5.444, 95% CI 0.653–45.386) and a two-sided Fisher's exact test (P = 0.159).

German Shepherds with mammary tumours had Hap0–17 samples, Hap1–4 samples, and Hap5–1 sample. The difference relative to the healthy group was not statistically significant. Among the group of diseased German Shepherds (n = 22), Hap0 (OR 10.200, 95% CI 2.742–37.945, P < 0.001) was more prevalent than in the remaining sick dogs (n = 28).

A group of animals with simple epithelial mammary tumours (n = 18) was selected to study the association between SNPs and the proliferation index (Ki-67). The immunohistochemical reaction for Ki-67 antigen was nuclear and showed a diffuse granular pattern. Weak staining was observed in the cytoplasm of individual epithelial cells, including cells with mitotic figures (Fig. 1). The lowest proliferation index (Ki-67) was noted in a group of animals with Hap0–15.95%±5.11 (2.97–42.16%) by comparison to the remaining sick animals with a mean value of the proliferation index of 24.09%±3.49 (4.67–49.29%). Each separate group (Hap1, Hap2, Hap3 and Hap4) had a higher proliferation index than the Hap0 group, except for Hap5 group with a mean value 11.63%.

Discussion

Two SNPs, 5'-UTR c.-42 and − 30, have not been described in the National Center for Biotechnology Information to date.

Compared to previous studies, SNPs c.-127 (promoter region) and c.-68 (5'-UTR) were identified, while the SNP at position c.-61 remained undisclosed. In healthy dogs, several polymorphisms within the promoter and 5'-UTR of the canine *GSTP1* gene, including single-nucleotide polymorphisms at positions c.-127, c.-68, c.-46, c.-43, c.-27, and c.-21, were identified by Sacco et al. [26]. The same SNPs were found in this study; however, polymorphisms at positions c.-52, c.-48, c.-42, c.-30, and deletions c.-46-44, c.-46-26, and c.-24-22, which are described in the current study, are different from those reported by other authors. Additionally, the SNP at position c.-37 was not found.

SNPs described by Sacco et al. [26] had minor allele frequencies that differed from those reported in the current study (Table 2). Only one SNP at position c.-127 had a lower MAF (0.009) than in the studied group of sick dogs. The remaining SNPs had higher MAFs: c.-68–0.131, c.-46–0.496, c.-43–0.138, c.-27–0.257, and c.-21–0.356.

By studying the promoter region of human *GSTP1*, Cauchi S. et al. [27] identified 3 SNPs at positions − 312, -317, and − 319. Coles F. et al. [28] identified polymorphisms − 105 T > C and − 88 A > G in the promoter region of human *GSTA1*, which result in reduced gene expression. Morrow et al. [29] reported the human

and rat *GSTP* promoter sequences for the transcription-activating factor AP-1 (TGACTCA) and the transcription regulatory protein Sp1 (GGGCGG). SNPs have not been discovered in similar sites in the proximal promoter of canine *GSTP1*, and the SNP − 127 G > C found in the current study does not belong to these elements.

A core promoter was present in the studied fragment. In humans, it encompasses the first 35 nucleotides proximal to the transcription start site and the entire 5'-UTR [30], and it contains elements such as the TFIIB recognition element (BRE), the TATA box, the initiator (Inr), motif ten element (MTE), and the downstream promoter element (DPE) [31]. These regulatory elements may be present in canine *GSTP1*, and polymorphisms may disrupt the regulation of their expression. Elements MTE and DPE are located in the 5'-UTR. MTE can occur at positions − 67 to -56, and it promotes transcription by RNA polymerase II [32]. MTE overlaps with DPE, which consists of 5 nucleotides and can be located at position − 62 to -52 [33, 34]. SNPs in the possible BRE, TATA box, Inr, and MTE regions of the gene were not revealed, but a polymorphism C > T at position c.-52, which alters the sequence of the possible DPE and may reduce TFIID binding and transcriptional activity, was identified.

In some genes, downstream core elements [35, 36] were identified that function similarly to DPE and are located between − 75 and − 35. The following SNPs: -68, -52, -48, -46, del.-46-44, -43, and − 42 may affect DPE function. The assumption is supported by the results of *in silico* analysis (Table 3), which demonstrated the impact of SNPs in Hap1, Hap2, Hap3, Hap4, and Hap5 on sites involved in transcription.

Radic et al. [37] found that the human *GSTO2* contains SNP A > G (rs2297235) in 5'-UTR which is associated with a 1.46-fold increased risk of clear cell renal cell carcinoma (95% CI: 1.02–2.09, P = 0.041) and higher levels of 8-OHdG, a biomarker of oxidative DNA damage (1.41 ng/mL vs. 0.99 ng/mL, respectively P = 0.042). This SNP is also associated with urothelial carcinoma in humans [38]. Ann M. Moyer et al. [39] found three SNPs in the 5'-UTR of *GSTP1* at positions − 20, -18, and − 1 in the Mexican American population. They studied the influence of 5'-UTR − 18 G > A on the reduction of *GSTP1* expression, and showed a significant association (P = 3.2 × 10^-6). This SNP was predicted to affect the AML-1a binding site and reduce mRNA expression.

Estaller et al. [40] investigated serum Ki-67 concentrations in dogs diagnosed with mammary tumours and reported a significant difference between dogs with malignant neoplasms and those with non-tumour-related diseases. Their findings suggest that serum Ki-67 may be a prognostic marker. The Ki-67 proliferation index was specifically evaluated within various types of malignant mammary tumours in the present study. Notably, the dogs included in the study had different haplotypes, which may further influence the observed variations in proliferation activity.

Renwick et al. [41] did a comparative analysis of Ki-67 proliferation indices and telomerase activity in lymphoid tissues from dogs with lymphoma and non-lymphomatous lymph nodes, demonstrating a correlation between these two parameters. Based on the authors' *in silico* analyses, specific polymorphisms may affect the transcriptional regulation of various proteins, and these transcriptional changes could, in turn, be associated with the Ki-67 proliferation index.

Since the proliferation index Ki-67, which is regarded as a prognostic tool for tumour malignancy, is significantly higher in samples Hap1-5 compared to Hap0, this may suggest that the presence of polymorphisms in the 5'-UTR of *GSTP1* in these samples could be considered important for diagnostic purposes. The detection of a polymorphism in the promoter region underscores the need for further investigation.

In conclusion, nine polymorphisms and three deletions within the *GSTP1* gene were first identified in dogs with mammary tumours. The most frequent were SNP – 21. A > G and deletion TGC c. -44-46. The differences between healthy dogs and the combined sick group (Hap1-6) were statistically significant. Furthermore, some diseased animals assigned to Hap1-5 exhibited a higher Ki-67 proliferation index compared with dogs in the Hap0 group. These findings suggest that these polymorphisms may serve as tumour biomarkers.

Declarations

Ethics approval and consent to participate

The study was reviewed and approved by the Committee on Animal Use and Ethical Review. Informed consent was obtained from the owners for the use of the animals in this study.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

O.F. conceptualized the study and designed the work, collected material, performed the main molecular genetic investigations (PCR and sequencing analysis) and wrote the manuscript.

N.P. collected material, performed the main molecular genetic investigations (PCR and sequencing analysis) and wrote the manuscript.

L.A. collected material, performed gene sequencing and critically revised the manuscript.

W.L. performed Ki-67 immunohistochemical analysis and critically revised the manuscript.

I.K. collected material, performed PCR and sequencing analysis.

O.Z. collected material and performed histopathological analysis.

S.W. conceptualized the study, critically revised the manuscript for important intellectual content and edited the final version.

All authors have read and approved the final manuscript.

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Table

Table 4 is available in the Supplementary Files section.

Figures

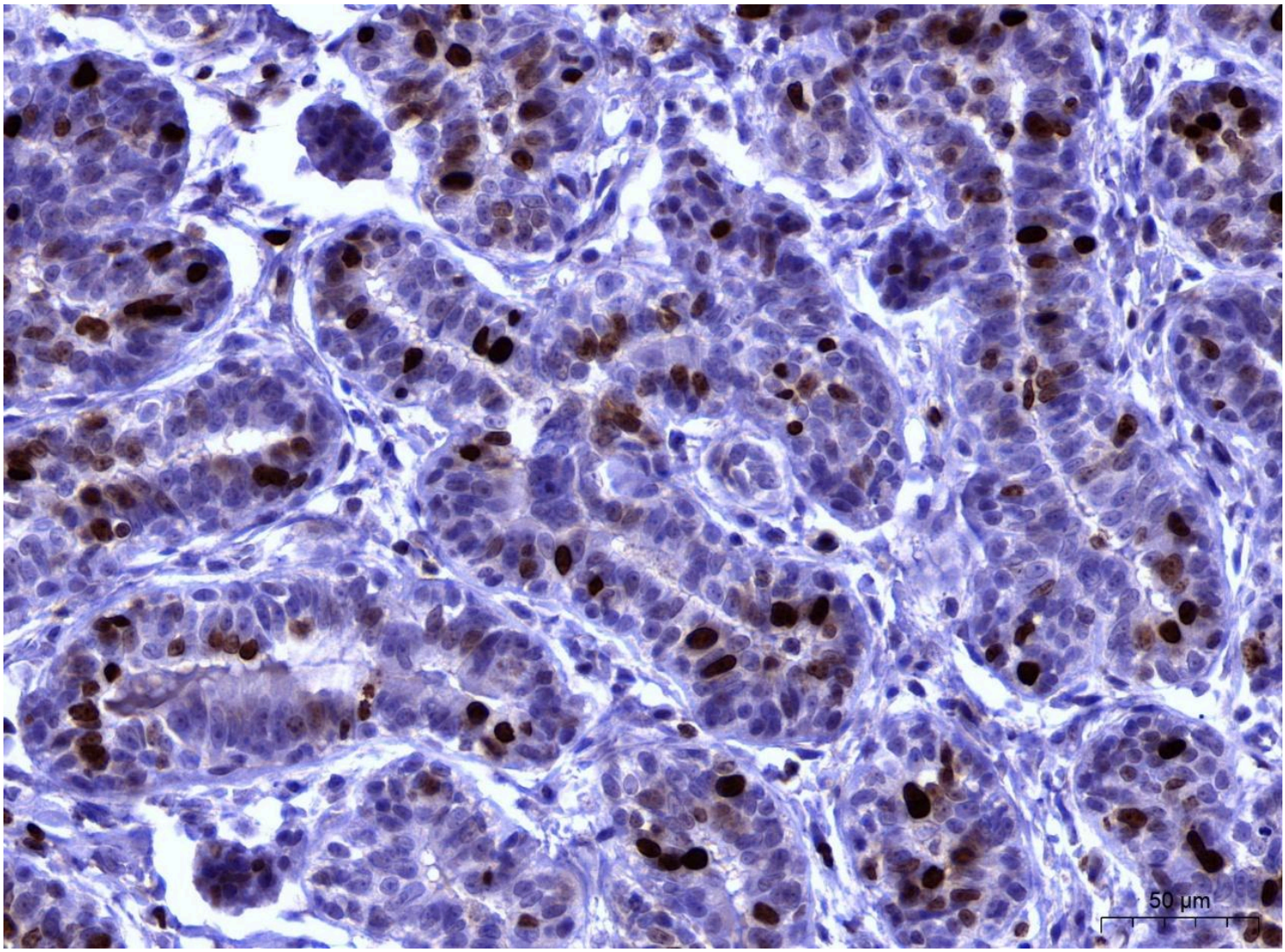


Figure 1

Moderate to strong immunohistochemical reaction to Ki-67 antigen in numerous tubulopapillary carcinoma cells. IHC staining, Mayer's haematoxylin counterstain. Bar=50 µm

Supplementary Files

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- Table4.docx