

## ORIGINAL ARTICLE

# Follicle-stimulating hormone receptors: A comparison of commercially-available monoclonal and polyclonal antibodies as immunohistochemical markers for cancer research

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## Summary

**Purpose:** In recent studies, follicle-stimulating hormone receptors (FSHRs) have been reported in a wide range of malignant and benign tumours, depending on the type of antibody used. Using two commercially available antibodies (monoclonal and polyclonal), the current research attempted to demonstrate the usefulness of each antibody for investigating FSHRs in non-canonical tissues. Further, we sought to replicate the results of a major study which demonstrated the presence of FSHRs in the endothelial cells of perineoplastic blood vessels.

**Methods:** Immunostaining was performed on 16 surgically excised benign and malignant tumor tissue samples using both monoclonal and polyclonal anti-FSHR antibodies.

**Results:** Positive staining of FSHRs was heterogeneous among the tissue samples used for analysis, and was confirmed not only in tumour and endothelial cells of perineoplastic blood vessels, but also in benign and normal cells.

Based on our findings, FSHR staining using a polyclonal antibody appeared to be highly sensitive, but with a relatively low specificity. Comparatively, immunoreactivity using a monoclonal antibody appeared to show high specificity, but relatively low sensitivity. Although the selected monoclonal antibody for FSHRs seemed to be more specific than the polyclonal variant, neither exhibited a high overall specificity. Neither of the antibodies assessed in the present research could replicate the results of the aforementioned major study.

**Conclusions:** In conclusion, neither of the two commercially available antibodies seem to be appropriate for investigating FSHRs in non-canonical tissues and, by extension, their role in carcinogenesis.

**Key words:** cancer, follicle-stimulating hormone receptor (FSHR), immunohistochemistry, monoclonal antibody, polyclonal antibody

## Introduction

FSH is essential for human reproduction and is a key hormone in oogenesis and spermatogenesis. FSH is secreted by the adenohypophysis, serving a fundamental role in the normal functioning of ovaries and testes. The proper function and action of FSH depend on its binding and activation of FSHRs.

FSHR is a transmembrane glycosylated protein and a member of the G protein-coupled receptor family [1,2]. Historically, these receptors were thought to be found only in ovarian granulosa cells and Sertoli cells [1,2]. Somewhat more controversial are reports of FSHRs in ovarian and testicular en-

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Received: 29/04/2018; Accepted: 21/05/2018

endothelial cells [3] and in the uterus [4]. Recently, their presence has been reported in a wide range of malignant and benign human proliferative conditions. Several groups of researchers have reported the presence of FSHRs in the endothelial cells of perineoplastic blood vessels [5,6]; using a highly specific, non-commercially available monoclonal antibody (FSHR323), they successfully showed that FSHRs are selectively expressed in malignant tumours, but are absent in normal, inflammatory and benign human tissues. According to their studies, FSHRs are present on the surface of endothelial cells of peritumoural blood vessels, both intra- and extratumourally, and in “shells” ranging in thickness from 7 to 15 mm. However, some groups of researchers, using commercially available polyclonal antibodies, have conducted similar studies yielding results that are both less specific and less sensitive,

as shown in our recently published review [7]. Until now, various groups of researchers have attempted to demonstrate and quantify the presence of this novel marker (FSHR) in malignant solid tumours, and to prove its supposed role in angiogenesis.

The present study attempted to replicate the results of Radu et al. [5], who demonstrated the presence of FSHRs in the endothelial cells of perineoplastic blood vessels, and to evaluate the possible usefulness of two commercial antibodies (one monoclonal and one polyclonal) in the immunohistochemical research of FSHRs in non-canonical tissues. We sought to perform immunohistochemistry on a wide range of normal, benign and malignant human tissues to assess differences and similarities between immunostaining results as well as between our results and those reported by other researchers.

**Table 1.** Tissue sample characteristics

<i>Tissue</i>	<i>Histopathologic type</i>	<i>Other characteristics</i>
Ovarian	normal tissue	pT3c N1 Mx V0, FIGO IIIC
	serous benign cystadenofibroma	
	high-grade serous carcinoma	
Testicular	normal tissue	pT1 Nx Mx L0 V0
	seminoma	
Thyroid	follicular adenoma	pT4a N1a Mx L1 V0
	papillary carcinoma	
Breast	normal tissue	triple-negative without neoadjuvant chemotherapy (Nottingham II, Ki-67=25%)
	fibroadenoma	
	invasive carcinoma NOS	
	invasive carcinoma NOS	
Skin	normal tissue	luminal A type with neoadjuvant chemotherapy (ypT2 N3a L1 V0 R0, Nottingham III, ER=80%, PR=0%, Her2=0, Ki-67=35%)
	malignant melanoma	
	benign fibrous histiocytoma	
Soft tissue	myxofibrosarcoma	luminal A type without neoadjuvant chemotherapy (pT1c N0 L0 V0 R1, Nottingham II, ER=90%, PR=90%, Her2=0, Ki-67=20%)

**Table 2.** Characteristics of anti-FSHR antibodies

<i>Product name</i>	<i>Description</i>	<i>Application</i>	<i>Immunogen</i>	<i>Positive control</i>	<i>Isotype</i>
anti-FSH receptor antibody (ab150557)	rabbit polyclonal	IHC-P	synthetic peptide, corresponding to 18 amino acids from the N-terminal extracellular domain of human FSH receptor	human ovary tissue	IgG
anti-FSH receptor antibody (FSHR/1400) (2492-MSM1-P0)	mouse monoclonal	IHC-P	recombinant full length protein corresponding to human FSH receptor	human uterine carcinoma tissue	IgG1

IHC-P: Immunohistochemistry on paraffin embedded tissues, IgG: Immunoglobulin G

## Methods

### Sample tissues

We examined 16 tissue samples of various benign and malignant tumours, obtained after surgical excision at “Prof. Dr. Ion Chiricuta” Institute of Oncology in Cluj-Napoca, Romania. All specimens were preserved in 10% buffered formalin, embedded in paraffin following routine histological techniques and diagnosed accordingly. The samples included are presented in Table 1.

The protocol of this study was submitted to the Ethics Committee of “Iuliu Hatieganu” University of Medicine and Pharmacy as well as to the Ethics Committee of “Prof. Dr. Ion Chiricuta” Institute of Oncology. Both Ethics Committees approved our study protocol.

### Measurements and definitions

We used a descriptive scale for characterizing the intensity of FSHR immunostaining: negative staining, weak staining, moderate staining and strong staining. Negative controls were created using analogous histological sections stained by the same technique with omission of the primary antibody. Positive controls were created using healthy human ovary tissue for the poly-

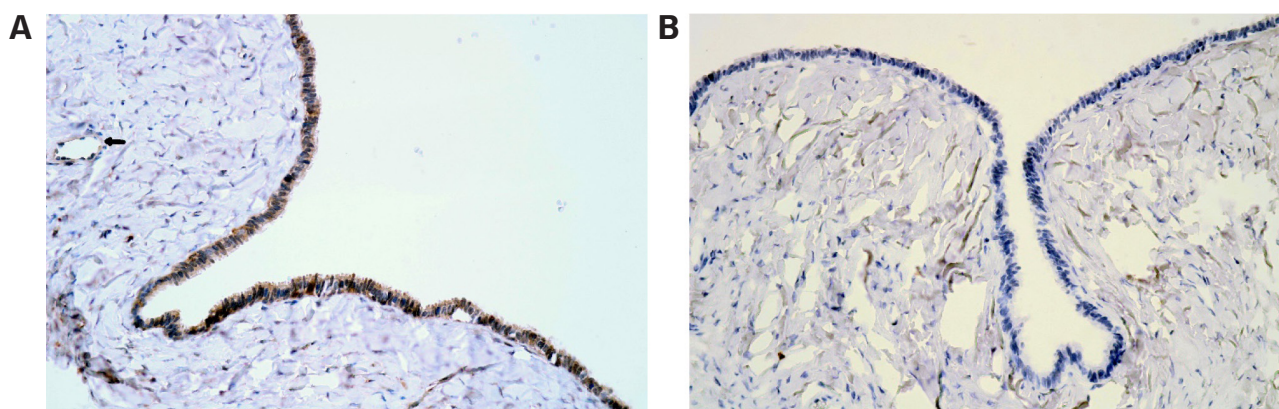
clonal antibody and human uterine carcinoma for the monoclonal antibody.

### Immunohistochemistry

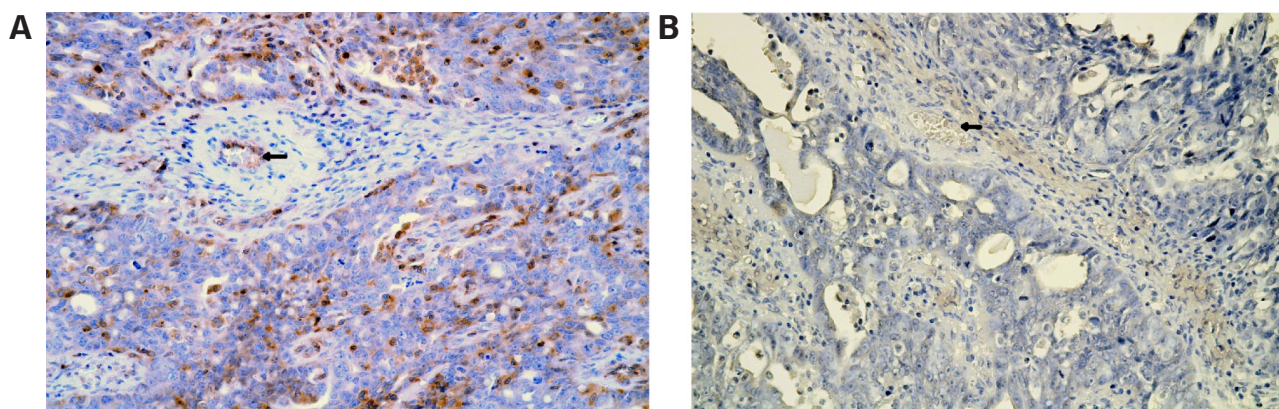
Immunostaining was performed on two successive, 4 mm thick paraffin sections taken from each of the 16 surgical specimens; one for staining with the polyclonal anti-FSHR antibody (Product ID: ab150557 – Abcam; Cambridge, Massachusetts) and the other for staining with the monoclonal anti-FSHR antibody (Product ID: 2492-MSM1-P0 – enquire BioReagents; Denver, Colorado). The characteristics of the aforementioned antibodies are presented in Table 2.

Using a NovoLink Max Polymer Detection System (Leica Biosystems; Newcastle, United Kingdom), an optimized protocol was developed by performing several immunostaining tests following the manufacturer’s instructions and making several researcher-defined modifications. The final protocol used in the present study is detailed below.

Tissue sections were attached to silanized slides and incubated at 37°C for 24 hrs. Samples were dewaxed in successive baths of xylene and ethanol, then washed twice in distilled water and rinsed with tris buffered saline (TBS) for 5 min. Heat-induced epitope retrieval



**Figure 1.** Evaluation of antibody staining in benign serous cystadenofibroma of the ovary (arrows indicate blood vessels). **A:** Immunostaining using the anti-FSHR polyclonal antibody (magnification 400×); **B:** Immunostaining using the anti-FSHR monoclonal antibody (magnification 400×).



**Figure 2.** Evaluation of antibody staining in papillary serous ovarian carcinoma (arrows indicate blood vessels). **A:** Immunostaining using the anti-FSHR polyclonal antibody (magnification 400×); **B:** Immunostaining using the anti-FSHR monoclonal antibody (magnification 400×).



was performed by boiling tissue sections for 20 min in 0.1 M sodium citrate buffer (pH 6.0), followed by cooling at room temperature for 20 min. Slides were then rinsed again with TBS for 5 min. Peroxidase activity was blocked using 3% hydrogen peroxide for 5 min; slides were rinsed again with TBS. Non-specific binding reactions were blocked with Protein Block Buffer for 5 min. Slides were rinsed again with TBS and then incubated with the Abcam polyclonal primary antibody (4 µg/mL) / enQuire BioReagents monoclonal primary antibody (4 µg/mL) in a humidity chamber at room temperature for 30 min. For improved penetration of the secondary reagents, slides were incubated with Post Primary solution for 25 min. Slides were then rinsed twice with TBS and incubated at room temperature with 100 µL of NovoLink Polymer solution for 25 min. Following incubation, sections were rinsed twice with TBS and incubated again at room temperature with 100 µL of 3,3'-diaminobenzidine (DAB) chromogen for 5 min. Each section was washed with distilled water, counterstained with hematoxylin, washed with tap water, rinsed in lithium carbonate solution and washed again with tap water. Finally, the slides were dehydrated in two successive baths of 96% ethanol and absolute ethanol, respectively, clarified in xylene for 5 min and then

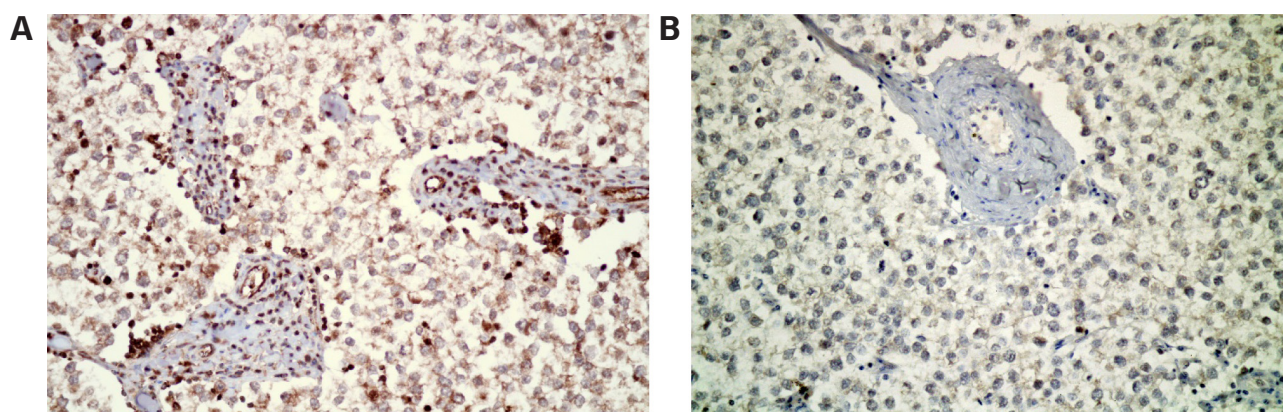
mounted using an automated film coverslipping machine.

Staining results were assessed by two pathologists using the aforementioned descriptive scale. For each tissue sample, a consensus was reached between the two specialists.

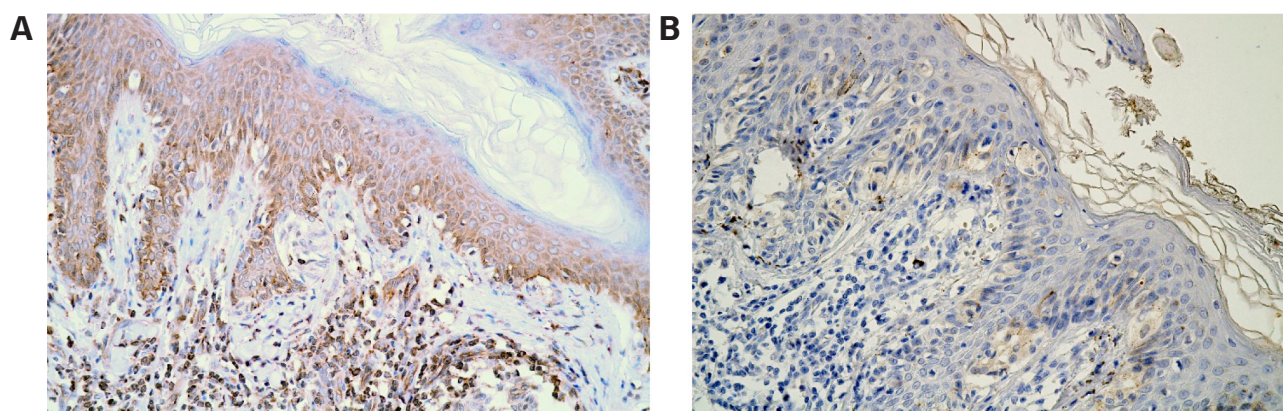
## Results

Due to the expected localization of FSHRs on certain tissues, antibodies were first tested and evaluated on normal ovarian tissue, pathologic ovarian tissue (Figures 1 and 2), and both normal and pathologic testicular tissue (Figure. 3).

In normal ovarian tissue, we observed strong results from FSHR immunostaining using the polyclonal antibody on cortical stroma, granulosa cells, theca cells, and on the endothelium of blood vessels. Conversely, using the monoclonal antibody, we observed weak positive staining of granulosa cells and theca cells; all other cell types were negative. Histiocytes were the only cell type with strong staining using both monoclonal and polyclonal antibodies.



**Figure 3.** Evaluation of antibody staining in testicular seminoma. **A:** Immunostaining using the anti-FSHR polyclonal antibody (magnification 400×); **B:** Immunostaining using the anti-FSHR monoclonal antibody (magnification 400×).



**Figure 4.** Evaluation of antibody staining in cutaneous malignant melanoma. **A:** Immunostaining using the anti-FSHR polyclonal antibody (magnification 400×); **B:** Immunostaining using the anti-FSHR monoclonal antibody (magnification 200×).



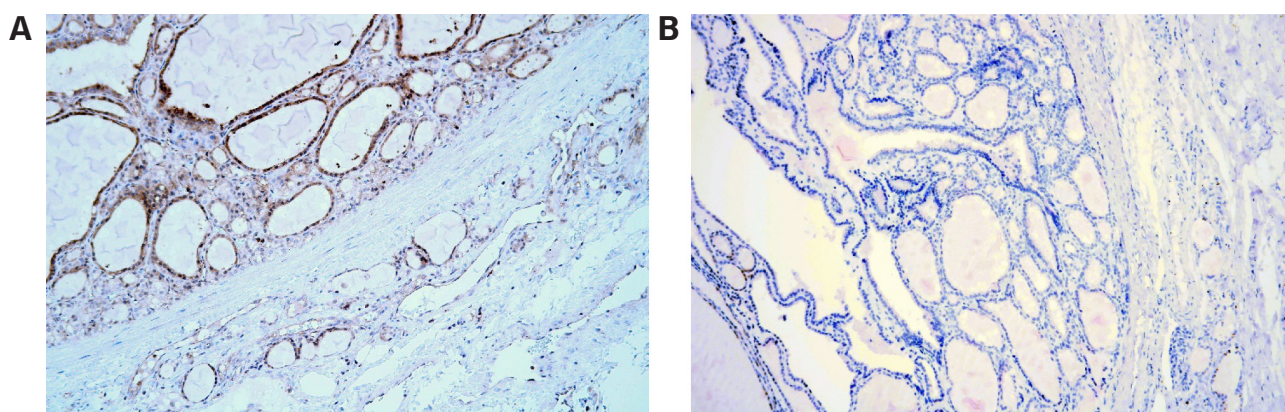
In pathologic ovarian tissue, we tested both antibodies on a benign tissue sample (serous cystadenofibroma – Figure 1) and a malignant tissue sample (high-grade serous ovarian carcinoma – Figure 2). Serous cystadenofibroma exhibited strong positive FSHR staining of the epithelial component and of the endothelium of blood vessels using the polyclonal antibody (Figure 1A), but negative staining using the monoclonal antibody (Figure 1B). High-grade serous ovarian carcinoma immunostained with the polyclonal antibody was negative on tumour cells, but was strong on histiocytes and moderate on the endothelium of blood vessels (Figure 2A). Furthermore, an identical staining was obtained using the monoclonal antibody, except for the endothelium, which exhibited negative staining (Figure 2B).

In normal testicular tissue, we observed that Leydig cells, Sertoli cells, germinal cells and the endothelium of blood vessels showed a strong positive immunoreaction with the polyclonal antibody and a weak immunostaining using the monoclonal antibody. Using a tissue sample of testicular semi-

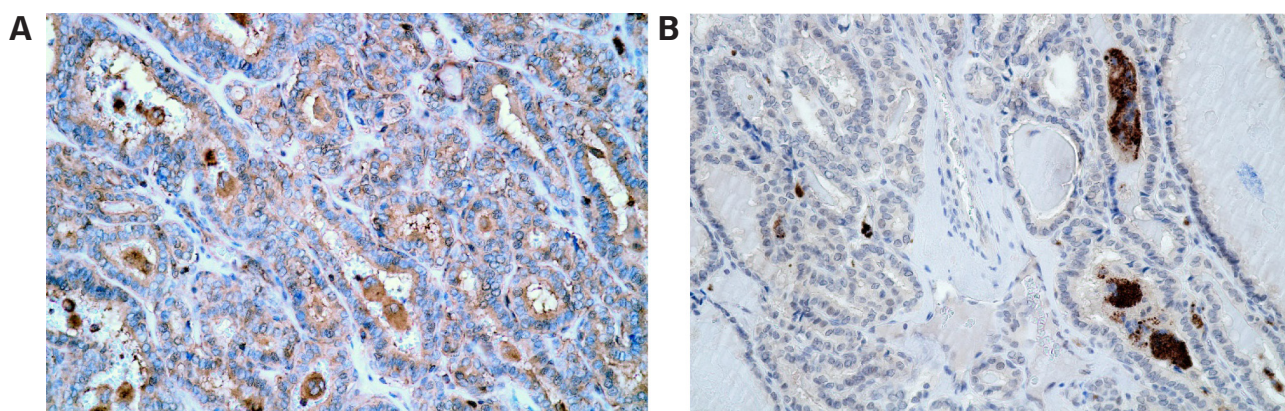
noma, we obtained results similar to those previously mentioned. The polyclonal antibody showed a strong immunostaining of Leydig cells, Sertoli cells, germinal cells and the endothelium of blood vessels (Figure 3A), but a weak immunoreaction using the monoclonal antibody (Figure 3B).

Following evaluation of expected FSHR localization on a subset of tissues, we performed tests on normal, benign and malignant tissues samples where FSHRs were not previously thought to be present. We conducted identical immunohistochemistry tests on tissues which recently published literature have suggested may contain FSHRs: skin tissue samples (Figure 4), thyroid tissue samples (Figures 5 and 6) and breast tissue samples (Figure 7).

Performing immunostaining with the anti-FSHR polyclonal antibody on normal skin tissue yielded moderate staining of the epidermis, and strong staining on inflammatory cells and on the endothelium of blood vessels. Comparatively, the anti-FSHR monoclonal antibody did not bind to any structure on the normal skin tissue sample. Using



**Figure 5.** Evaluation of antibody staining in thyroid adenoma. **A:** Immunostaining using the anti-FSHR polyclonal antibody (magnification 200x); **B:** Immunostaining using the anti-FSHR monoclonal antibody (magnification 200x).



**Figure 6.** Evaluation of antibody staining in papillary thyroid carcinoma. **A:** Immunostaining using the anti-FSHR polyclonal antibody (magnification 400x); **B:** Immunostaining using the anti-FSHR monoclonal antibody (magnification 400x).



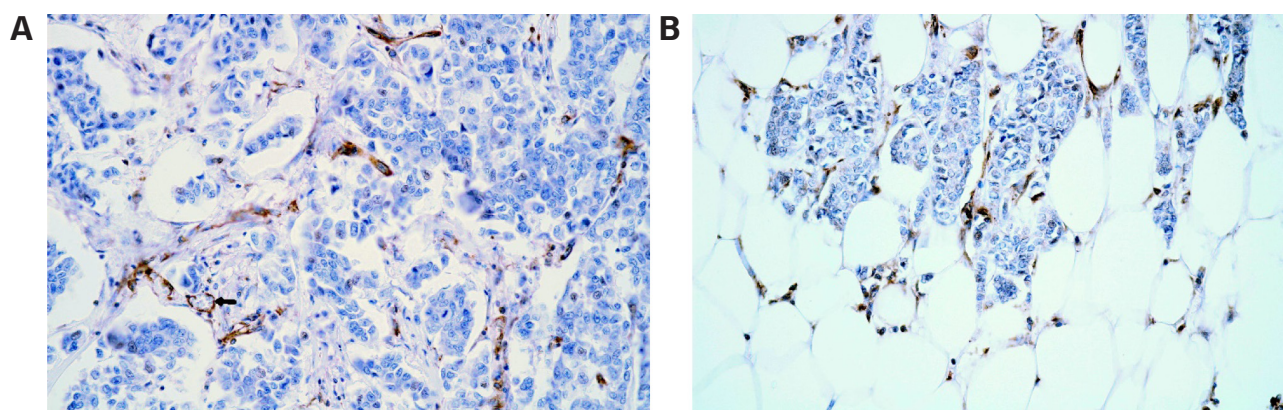
a tissue sample of cutaneous malignant melanoma, the anti-FSHR polyclonal antibody presented strong staining of inflammatory cells and moderate staining of the epidermal basal layer (Figure 4A); negative staining of tumour melanocytes was observed. The monoclonal antibody revealed negative staining of tumour cells, with the exception of strong staining of histiocytes (Figure 4B).

Thyroid adenoma tissue showed strong polyclonal anti-FSHR immunostaining at the apical poles of cells and in inflammatory foci (Figure 5A), however monoclonal antibody immunostaining was negative (Figure 5B). Moderate staining was obtained on the endothelium of blood vessels when using the polyclonal antibody (Figure 5A). The anti-FSHR polyclonal antibody revealed a uniform, moderate staining on papillary thyroid carcinoma (Figure 6A). Results of immunostaining were negative when using the monoclonal antibody (Figure 6B) except for histiocytes, which showed a strong immunoreaction in both tissue samples.

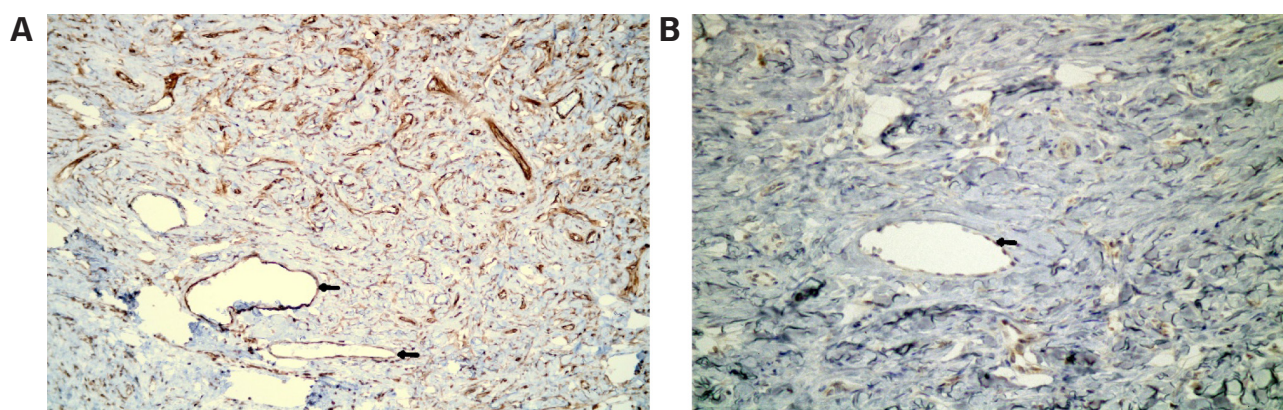
Investigating the normal and benign tumour breast tissue (fibroadenoma), we observed negative staining using the monoclonal antibody. Using the polyclonal antibody in normal breast tissue, moderate staining of the ductal epithelium and of the basal myoepithelial cells was noted. We also identified weak staining of epithelial cells of acini. Strong staining of the epithelium and moderate staining of the endothelium of blood vessels were also observed in the fibroadenoma tissue sample using the polyclonal antibody.

Breast cancer tissue samples consisted of one triple-negative invasive breast carcinoma, one luminal A type invasive breast carcinoma with neoadjuvant chemotherapy (Figure 7A) and one luminal A type invasive breast carcinoma without neoadjuvant chemotherapy (Figure 7B).

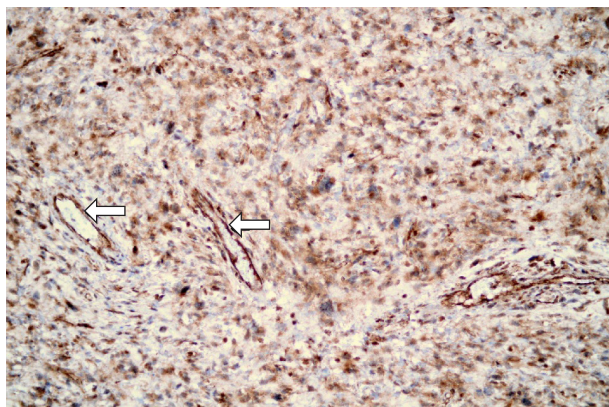
The triple-negative invasive breast carcinoma without neoadjuvant chemotherapy immunostained with anti-FSHR polyclonal antibody revealed weak staining of tumour cells and a strong



**Figure 7.** Evaluation of antibody staining in 2 invasive breast carcinomas (1 luminal A type without neoadjuvant chemotherapy and 1 luminal A with neoadjuvant chemotherapy) (arrow indicate blood vessels. Immunostaining using the anti-FSHR polyclonal antibody (magnification 400×). **A:** With neoadjuvant chemotherapy; **B:** Without neoadjuvant chemotherapy.



**Figure 8.** Evaluation of antibody staining in benign fibrous histiocytoma (arrows indicate blood vessels). **A:** Immunostaining using the anti-FSHR polyclonal antibody (magnification 200×); **B:** Immunostaining using the anti-FSHR monoclonal antibody (magnification 400×).



**Figure 9.** Evaluation of antibody staining in myxofibrosarcoma (arrows indicate blood vessels) - immunostaining using the anti-FSHR polyclonal antibody (magnification 200×). Arrows indicate blood vessels.

positive immunoreaction on the endothelium of peripheral blood vessels. The invasive breast carcinoma luminal A type with neoadjuvant chemotherapy and the invasive breast carcinoma luminal A type without neoadjuvant chemotherapy immunostained with anti-FSHR polyclonal antibody also revealed weak, almost negative, staining of tumour cells (Figure 7). Furthermore, the polyclonal antibody showed strong positive immunostaining of the endothelium of blood vessels belonging to the invasive breast carcinoma luminal A type with neoadjuvant chemotherapy (Figure 7A) compared to negative staining in the breast carcinoma without neoadjuvant chemotherapy (Figure 7B). The monoclonal antibody showed negative staining on all three breast tissue samples.

Finally, we performed immunoreaction tests on a benign fibrohistiocytic tumour (Figure 8) and a malignant fibroblastic tumour (Figure 9). Using the polyclonal antibody on a benign fibrous histiocytoma tissue sample, strong staining of inflammatory cells and of the endothelium of blood vessels was observed (Figure 8A), compared to a weak immunoreaction in the same structures (inflammatory cells and endothelium) when using the anti-FSHR monoclonal antibody (Figure 8B). The myxofibrosarcoma tissue sample stained with the anti-FSHR polyclonal antibody exhibited a strong immunoreaction on tumour cells, of both the inflammatory foci and the endothelium of blood vessels (Figure 9). Comparatively, the monoclonal antibody showed negative staining with weak immunoreaction of the inflammatory foci.

## Discussion

Numerous studies over the past 10 years have highlighted the role of biomarkers in the diagnosis

and prognosis of various cancers, their role in tumour growth and angiogenesis, and in the microenvironment of neoplastic cells [5]. The present study sought to contribute to our collective scientific understanding of the proliferation of FSHRs throughout a variety of tissues where these receptors were previously thought to be absent. We sought to begin scholarly conversations surrounding several key questions: Can the tested commercial anti-FSHR antibodies become a valid immunohistochemical marker for cancer research? Are these two commercially available antibodies reliable enough to test FSHRs in non-canonical tissues?

Our goal was to determine if the tested antibodies could mirror highly specific and promising results obtained by previous research [5] using a commercially unavailable antibody. Our results are quite similar to those described by other researchers who used different polyclonal anti-FSHR antibodies, and we also observed similarities to results obtained using FSHR323 [5], similarities which we believe are worth noting. Furthermore, utilizing a new, commercially available anti-FSHR monoclonal antibody in our study, we observed a moderate degree of specificity in our results, however failed to observe the level of specificity presented by Radu and Ghinea [5].

FSHRs and their role in ovarian cancer have been studied by several groups, such as Heubner et al. [8], Ludwig et al. [9] and Zhang et al. [10], both on tissue samples and cell lines. They concluded that FSHRs may play a role in ovarian cancer. The first two groups applied genotyping in lieu of immunohistochemistry, and the third group used Western blot analysis (anti-human FSHR rabbit polyclonal antibody; Labvision Corporation/NeoMarkers, Fremont, CA). Without providing detailed information regarding the anti-FSHR antibody used in their research, and given that it was published before the Radu and Ghinea study [5], we can not properly compare similarities or differences between these studies. Our results show that when using a polyclonal antibody, moderate to strong immunostaining can be obtained, even on the endothelium of blood vessels of normal and pathologic tissue. The monoclonal antibody failed to show any specific binding to the endothelium of blood vessels belonging to normal, benign or malignant tissues. In comparison with FSHR323, our results show that the selected polyclonal antibody can exhibit high sensitivity but low specificity, while the monoclonal antibody exhibits the inverse behavior: high specificity and low sensitivity.

To our knowledge, thyroid tissue samples have thus far been tested using only commercially available anti-FSHR antibodies [11–13]. In their study



Liu et al. [11] used a FSHR kit from Boster Biological Technology (Wuhan, China) and the other two studies of Pawlikowski et al. used a rabbit anti-human FSHR polyclonal antibody raised against a 1–190 amino acid sequence from the human FSH-R (sc-13935; Santa Cruz Biotechnology Inc.). Liu et al. showed the presence of FSHRs in the cytoplasm of thyroid epithelial cells both in normal and neoplastic thyroid tissue samples; the undifferentiated thyroid carcinoma showed a negative staining. In comparison, Pawlikowski et al. [12,13] showed findings similar to those of the present study when using the polyclonal antibody. According to the authors, FSHRs were present in some of the thyroid follicular adenomas and present in almost all neoplastic cells. They reported positive immunostaining of the endothelium of blood vessels belonging to neoplastic tumors and follicular adenomas in almost half of the tissue samples analyzed. The use of a monoclonal antibody in our study showed a negative immunoreaction in both thyroid adenoma and thyroid neoplasia. The positive staining of histiocytes observed in our study were not reported in any of the aforementioned studies. To the best of our knowledge, the medical literature does not report any study of thyroid tissues immunostained with FSHR323 antibody [5].

The results of polyclonal immunostaining of the breast tissue samples in our study are particularly noteworthy. Our findings indicate a strong immunoreaction of the endothelium of perineoplastic blood vessels in invasive breast carcinoma treated with neoadjuvant chemotherapy and a negative immunostaining in breast carcinoma without neoadjuvant chemotherapy. These observations are rather descriptive and opportunistic due to the lack of a larger series of breast tissue samples. We also observed weak staining of tumour cells when using the polyclonal antibody. However, when using our anti-FSHR monoclonal antibody, we obtained negative results on all breast cancer tissue samples. We are unaware of any other studies performed on breast tissue samples utilizing commercially available anti-FSHR antibodies.

Regarding the malignant melanoma tissue samples, we were unable to identify any published studies regarding the presence of FSHRs in skin or melanoma using the antibody of Radu [5] or other commercially available anti-FSHR antibodies. Here, we report positive immunostaining of normal skin and cutaneous malignant melanoma using an anti-FSHR polyclonal antibody; we observed no staining, except in select histiocytes, when using the monoclonal antibody. We can not draw any conclusions about the presence of FSHRs in normal skin or malignant melanoma due to the

lack of evidence using highly specific and reliable antibodies.

Renner et al. [6], using FSHR323, concluded that FSHRs are present in the endothelial cells of the peritumoural vasculature of soft tissue sarcomas, including myxofibrosarcoma. Our results using the polyclonal antibody showed not only a strong immunoreaction of the endothelium of blood vessels, but also of the tumour cells and of the inflammatory foci. However, negative staining was observed using the monoclonal antibody.

To date, only one study attributed to Maclellan et al. [14], using a commercially available anti-FSHR polyclonal antibody (polyclonal rabbit anti-human follicle-stimulating hormone receptor, GTX71309; GeneTex, Irvine, California), showed somewhat similar results with those of the studies using FSHR323. No positive immunostaining for FSHR was reported for the normal tissue samples. However, the nature of the non-malignant pathologic tissues samples used for this study brings the results into question.

Discordant results reported in the medical literature can be explained by dissimilar methods used for detecting FSHRs involving different commercially available antibodies [11–13], different types of tumours with distinct origin and evolution [15] and the potential existence of FSHR isoforms. There have been four isoforms identified (FSHR1, FSHR2, FSHR3 and FSHR4) and only FSHR1 and FSHR3 are currently known to have biological functions [16–18]. A study conducted by Urbanska et al. [19] even stated that “we were unable to detect FSHR protein in the blood vessels of human or mouse cancers by immunohistochemical analysis using commercially available antibodies”.

The supposed presence of FSHRs in the endothelium of blood vessels surrounding malignant tumours may present a promising target in the field of cancer research. Their possible role in the angiogenesis of cancer, and potential clinical implications, remain to be determined. In order to challenge this subject and introduce novel research findings, it is necessary to make use of highly specific antibodies. Presently, only studies which used FSHR323 [5] appear to show reliable data and results regarding the presence and role of FSHRs in carcinogenesis. Using commercially available antibodies, which seem unfit for current research, and in the absence of studies providing exact specifications for antibodies used, research in this area may be subject to erroneous comparisons and conclusions. We therefore wish to emphasize the importance of using reliable antibodies when initiating research endeavors to investigate the role of FSHRs in carcinogenesis.



In conclusion, immunostaining of FSHRs is currently quite challenging, complicated by an inadequate balance between specificity and sensitivity of the two commercially available antibodies which were tested. Their utility for investigating FSHRs in non-canonical tissues, and by extending their role in carcinogenesis, is currently suboptimal and we cannot recommend either of the two commercially available antibodies. Presently, understanding the exact role of these receptors in carcinogenesis using immunohistochemistry may only be pragmatically investigated with a highly specific antibody (FSHR323). Future research endeavors should seek to further investigate FSHRs using a highly specific monoclonal antibody such as FSHR323. Such an antibody, not available for commercial use, could be quite promising in terms of correlations with

other cancer-related factors already known for their utility in diagnosis and prognosis.

## Acknowledgements

The authors would like to thank Alexander Bogdan and Laura Sabau for proofreading and improving this manuscript. This study was funded by "Iuliu Hatieganu" University of Medicine and Pharmacy from Cluj-Napoca, Romania, based on PhD contract number 4003/01.10.2016 and research funding for PhD contract number 1300/7/13.01.2017.

## Conflict of interests

The authors declare no conflict of interests.

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