Immunohistochemical characterization of selected breast lesions

Ph.D. Thesis

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2016

LIST OF FULL PAPERS THAT SERVED AS THE BASIS OF THE PH.D. THESIS

I. **Kővári B**, Rusz O, Schally AV, Kahán Z, Cserni G.
Differential immunostaining of various types of breast carcinomas for growth hormone-releasing hormone receptor - Apocrine epithelium and carcinomas emerging as uniformly positive.
APMIS 2014;122:824-831.
IF: 2.042

II. **Kővári B**, SzászAM, Kulka J, Marusić Z, Sarcevic B, Tiszlavicz L, Cserni G.
Evaluation of p40 as a myoepithelial marker in different breast lesions.
Pathobiology 2015;82:166-171.
IF: 2.480

III. **Kővári B**, Báthori Á, Cserni G.
CD10 immunohistochemical expression in apocrine lesions of the breast.
Pathobiology 2015;82:259-263.
IF: 2.480

1. INTRODUCTION

1.1. THE INVOLVEMENT OF GROWTH HORMONE-RELEASING HORMONE (GHRH) IN CARCINOGENESIS

Growth hormone-releasing hormone (GHRH) has been implicated in carcinogenesis as a growth factor acting both indirectly through the neuroendocrine axis, and more significantly directly through autocrine and paracrine mechanisms.Many cancers of extrapituitary tissues, express GHRH and GHRH receptors (GHRH-R). The presence of GHRH and GHRH-R have also been documented in breast cancer. As evidence of an autocrine/paracrine regulatory mechanism, it has been shown that the knocking down of the GHRH gene expression in breast cancer cell lines results in reduced cellular proliferation. The transfection of the MCF-7 cells (originally devoid of GHRH-R) with the GHRH-R, results in increased cellular proliferation after the addition of exogenous GHRH. The transfection of MCF-7 cells with GHRH-R results in increased proliferation even without the addition of exogenous GHRH, suggesting a GHRH-independent activation. Furthermore, GHRH antagonists have been found to be effective in the reduction of invasive and metastatic potential of human cancer cell lines in vitro by modifying cellular adhesion, migration and survival. The antagonistic analogs of GHRH have been reported to consistently reduce the growth of several breast cancer models, and therefore such antagonists have been proposed as potential targeted therapeutic agents for breast carcinoma.

1.2. THE p53 TUMOR SUPRESSOR GENE FAMILY

Transformation-related protein 40 (p40) is one of several important transcription factors coded by the p53 tumor suppressor gene family. Transformation-related protein 63 (p63) is an other member of this gene family, and is utilized primarily as a marker of squamous, myoepithelial (MEC), prostate basal and urothelial cellsin current surgical pathology practice.p40 is the newest member of the family being used as an immunohistochemistry (IHC) marker and is reported to be superior to p63 for squamous differentiation in the differential diagnosis of non-small cell lung cancer.Many myoepithelial markers are also expressed in a group of breast carcinomas representing a basal-like nature or myoepithelial differentiation. Although

compared to cytokeratin 5 (CK5), p63 is only infrequently expressed by basal-like breast cancers (BLBC), there are only scant data on the expression of p40 in these tumors.

1.3. EXPRESSION OF CD10 IN BREAST TISSUE

In diagnostic breast histopathology, cluster of differentiation 10(CD10) IHC is usedto identify MECs. Although MECs around normal structures (ducts and lobules) are nicely highlighted by this marker, in pathologic conditions such as ductal carcinomain situ (DCIS), CD10 has a relatively low sensitivity as a MEC marker, and its specificity also seems compromised by the fact that, rarely, tumor cells also stainwith the antibody, although the pattern of staining in the neoplastic mammary epithelium has not been widely studied.Apocrine epithelium has been described to be positive for CD10, and Kalof et al. clearly documented the consistent luminal staining of apocrine metaplasia. While studying breast lesions immunostained for CD10 as a MEC marker, we also recognized that paratumoral apocrine cysts demonstrated a strong, predominantly apical reaction, and no previous studies have systematically examined CD10 expression of apocrine lesions.

2. AIMS

The aims of the present thesis are listed as follows:

To analyze aseries of breast carcinomas for the expression of GHRH-R and tocorrelate the presence of these receptors to histological features and morphological or biological subtypes of breast cancers.

To investigate a series of apocrine breast carcinomas for the expression of GHRH-R, because of the positive immunostaining of paratumoral benign apocrine epitheliumnoted during the course of the study.

To test the maintenance of GHRH-R status of the primary tumors in lymph node metastases.

To compare the expression of p40 versus p63 in the MEC component of normal breast structures and in breast lesions with occasional absence of or decrease in the staining for some other MEC markers and to see whether p40 was also superior to p63 as a MEC marker.

To assess and compare the expression of p63 versus p40 in triple-negative breast cancers(TNBC) showing CK5 expression, i.e. in tumors that would be classified as BLBCs by the surrogate IHC based approach.

To analyze a series of breast lesions with apocrine differentiation for the expression of CD10, both in the epithelial and the myoepithelial components and to explore how the immunostaining varied in benign, in situ and invasive malignant lesions.

3. MATERIALS AND METHODS

The conducted GHRH-R, p40 and CD10 expression related research was all carried out retrospectively using IHC.Formalin-fixed and paraffin-embedded tissue blocks obtained either from breast conserving surgery or total mastectomy specimens from the archives of theBács-Kiskun County Teaching Hospital,University of Szeged, University of Turin, and the 2nd Department of Pathology of Semmelweis University, Budapest were used.Composite tissue microarray (TMA) blockswere also built up from the donor blocks of multiple breast cancercases. Primary antibodies used in the different studies are listed as follows:GHRH-R: polyclonal (ab 76263);Abcam (Cambridge, UK), dilution: 1:250.p40: monoclonal (clone BC28);BioCare (Concord, USA), dilution: 1:200.CD10: monoclonal (clone 56C6);Cell Marque (Rocklin, USA), dilution: 1:50.

3.1. THE EXPRESSION OF GHRH-R IN DIFFERENT TYPES OF BREAST CARCINOMAS

Groups of different histological, molecular and clinicopathological types of breast cancer were selected. Small breast carcinomas (preferentially ≤2 cm) were included in the studyto limit the effect of tumor heterogeneity.Histological types included invasive tubular, invasive ductal / (no special type) NST and invasive lobular carcinomas (ILC) as defined by the World Health Organization (WHO)classification of breast tumors. Grading was performed on the basis of the Nottingham scheme.Molecular types were determined by means of the surrogateIHC-based approach as proposed by the St Gallen consensus meeting. Cases with casting-type microcalcification on themammogram were also included in the study becausethese tumors have been reported to have an unfavorableoutcome.During the analysis of the cases, we observed a consistent and strong staining for GHRH-R in foci of apocrine metaplasia. To investigate this unanticipated phenomenon, we included 31 cases of carcinomas with apocrine differentiation.We defined apocrine differentiation by using both histomorphologic and IHC criteria (estrogen receptor (ER) and progesterone receptor (PR) negativity, androgen receptor (AR) and gross cystic disease fluid protein 15positivity).Metastatic tumors of lymph node positive cases were also evaluated with TMA technique.Inall the selected lesions were evaluated using a lower and higher cutoff level of 10% and 50% of tumor cell positivity.

3.2. p40 EXPRESSION IN BASAL-LIKE BREAST CARCINOMAS AND p40 AS A MYOEPITHELIAL MARKER IN BREAST LESIONS

Groups of different histological types of breast lesions documented to demonstrate occasional alteration of MEC phenotype, including benign sclerosing lesions, DCIS and adenomyoepithelial lesions (AME) were randomly selected on the basis of their diagnoses, and associated normal breast tissue was analyzed.Randomly selected consecutive TNBCs expressing CK 5, corresponding to a subset of BLBCs on the basis of the surrogate IHC approach described by Nielsen et al were used to build up a TMA block.

3.3. CD10 EXPRESSION IN APOCRINE LESIONS OF THE BREAST

50 apocrine breast lesions were randomly selected including benign, in situ and invasive lesions. IHC stainings were carried out on 44 whole tissue sections and a TMA composite block.

4. RESULTS

4.1. THE EXPRESSION OF GHRH-R IN DIFFERENT TYPES OF BREAST CARCINOMAS

GHRH-R positivitywas detected in 54/100and 28/100 of the cases using 10% and 50% cut-off values, respectively.ILCs displayed GHRH-Rpositivity significantly more often (10% cut-off: p = 0.03; 50% cut-off: p = 0.0003)than ductal/NST carcinomas.Interestingly,the highest proportion of tumors demonstratingGHRH-R positivity was seen in grade 2 carcinomas. Statistical analysis of GHRH-R expression in different tumor grades failed to give a significant result (p = 0.0527) when using the 10% cut-off, but it was possible to get significant result applying the 50% cut-off level (p=0.001).To assess the relation ofGHRH-R expression and proliferation, on one hand the mitoticscore was used, but no association was found. On the other hand, the statistical analysis of Ki-67 labeling indices(LI)using the 50% cut-off yielded a significant difference (10% cut-off: p =0.0934; 50% cut-off: p = 0.0455). There wasno statistically significant association between nodal statusand GRHR-R

staining.As concerns the molecular types according to the IHC based classification (10% cut-off: p =0.009; 50% cut-off: p =0.00001),the luminal B-like category emerged as the molecular subtype with the highest ratio of positive cases.A substantial number (8/26, 31%) of triple-negative cases showed GHRH-R positivity in 10-50 % (average: 25%) of the tumor cells, but there were no cases (except the apocrine carcinomas) exceeding the 50% cut-off level.As a special clinical entity, 12 tumors with casting-type microcalcifications on the mammogram werealso included in the study. Although a higher percentage of these casesshowed GHRH-R positivity compared to NST carcinomas without casting type calcification, the statistical analysis showed no significant correlation. The striking majority of breast carcinomas with apocrine differentiation (10% cut-off: 97%, 50% cut-off: 90%) showed strong GHRH-R positivity.Lymph node metastases were only available for testing intwentypreviously examined GHRH-R expressing primary node positive tumors. Only a single case proved to be totally negative, and 70% (14/20) of the cases showed positivity in more than 10% of the tumor cells, whereas 30% (6/20) in more than 50% of the tumor cells.

4.2. p40 EXPRESSION IN BASAL-LIKE BREAST CARCINOMAS AND p40 AS A MYOEPITHELIAL MARKER IN BREAST LESIONS

Nineteen CK5-expressing TNBCs and thirty-six breast lesions with frequently altered MEC phenotype (10 AME, 13 high-grade DCIS with attenuated MEC layer and 11 sclerosing lesions) were included in this study, and normal breast tissue was also evaluated in each case, where available. In all the cases (31/31), a diffuse strong nuclear p40 positivity was detected in normal terminal ductulolobular units (TDLU). p40 and p63 staining patterns showed no difference in regular TDLUs.All AME showed nuclear p40positivity in the MEC component ranging from weakfocal (5/10) to strong diffuse (5/10). No conspicuousdifference between p40 and p63 reactivity was noted.The attenuated MEC around DCIS showedweaker staining compared with surroundingnormal TDLU, and negative cells with unequivocalMEC morphology were also detectable. Rarely,ducts affected by DCIS showing no positivity of theMEC were also recognized. In this set of lesions, MECstained identically with p40 andp63. All 11 sclerosing lesions displayed identicalp40 and p63 positivity of inconstant intensity, which was usually weaker than in the endogenous normal TDLUs serving as control. Focally negative MEC were also visible in multiple cases.

Of the 19 CK5-expressing TNBCs, 8 showed some p63 positivity, ranging from a few cells to 70% of the tumor cells. The intensity was generally weak and required scrutinous search. In contrast, p40 positivity could be seen in the majority of the cases (18/19) ranging from <1% to 70%. The intensity was either similar to that seen with p63 or stronger.

4.3. CD10 EXPRESSION IN APOCRINE LESIONS OF THE BREAST

Fifty apocrine lesions were included in the study: 10 cysts with or without papillary hyperplasia, 1 cyst without a MEC layer, 6 apocrine adenoses, 2 papillomas, 13DCIS, 14 invasive ductal/NST carcinomas and 4 ILCs. 17/19 [0.89; 95% CI 0.68–0.97] benign apocrine lesions showed complete or partial luminal CD10 staining, although most cases included parts without staining and 2 lesions were completely negative. The MECs in benign lesions were often but not always positive.

As concerns malignant lesions, 8/13 apocrine DCIS cases displayed no luminal staining, but 4 (0.31; 95% CI 0.13–0.58) demonstrated very focal luminal positivity. The MECs around the DCIS showed a spectrum of staining from nil to strong complete. Only 4/18 (0.22; 95% CI 0.09–0.46) invasive carcinomas demonstrated membranous staining. Cytoplasmic CD10 positivity was seen focally in 4 invasive cancers and in 3 DCIS, and more markedly in 1 invasive carcinoma NST; 2 invasive and 1 in situ carcinoma with ‘aberrant’ cytoplasmic staining demonstrated no membranous staining. Benign lesions showed membranous staining more commonly than malignant ones (17/19 vs. 8/31; $p < 0.0001$) and this was also true for aberrant cytoplasmic labeling (17/19 vs. 11/31; $p = 0.0006$).

5. DISCUSSION

5.1. THE EXPRESSION OF GHRH-R IN DIFFERENT TYPES OF BREAST CARCINOMAS

The endocrine effect of GHRH on cancer is dominantlymediated by autocrine/paracrine stimulation. GHRH antagonists have been tested as potential targeted therapeutic agents in several malignancies, including breast cancers. The incidence of GHRH-R expression in different breast cancer subtypes has not yet been

investigated extensively. Since the presence of the GHRH-R could be a selection criterion for potential treatment targeting the GHRH-R, it was thought that a study identifying potential subsets of tumors preferentially expressing the receptor could be of relevance.

As concerns the different histologic types of breast cancer, ILCs were significantly more frequently positive for GHRH-R than ductal/NST carcinomas.

Regarding the grade of differentiation, significant association with the GHRH-R status was just found using the 50% cut-off value, and grade 2 tumorsseemed to show GHRH-R positivity more frequentlythan grade 1 or 3 tumors. The reasons for this letter finding are not clear, and could be coincidental, especially in the light of molecular studies. Gene expression profile-based genomic grades match histological grades 1 and 3, but breast tumors classified as histological grade 2 fallinto either the low or high genomic gradecategory. Our results suggest that GHRH-R positivity can occur in any grade of breast cancer. In keeping with the results relating to the differentiation of the carcinomas, an ambiguous relation was found with proliferation depending on whether assessed by mitotic scores or the Ki-67 proliferation marker. Although there was no association between GHRH-R expression and mitotic scores, a significant correlation was found using the Ki-67 LIs. The significant association of tumor grade with the GHRH-R status using the 50% cut-off and the differences between the statistical analysis of mitotic scores and Ki-67 LIs suggest that the equivocal results may be due to the shortcomings of conventional histological grading, and maybe a stronger correlation could be found using genomic grades.

The study also incorporated 12 ductal/NST carcinomas with casting-type microcalcification on the mammogram. Our experience supports the poor outcome of these tumors, and this is why such cases were separately studied for their GHRH-R expression. Using the 50% cut-off,GHRH-R positivity was observed in 33% of the cases, which is more than double of the 14% positivity rate of ductal/NST carcinomas without casting-type microcalcification; however this difference failed to be statistically significant.

GHRH-R positivity was seen in all molecular types of breast cancer, including ER-positive and ER-negative cases. The majority of the luminal B-like tumors demonstrated strong and diffuse immune reaction with anti-GHRH-R, but as even luminal B-like tumors are heterogeneous, the significance of this finding is uncertain. Even though non-apocrine TNBCs showed GHRH-R positivity in a relatively low

percentage of tumor cells (5-30%, average: 15%) and cases (31% using 10% cut-off), the unfavorable prognosis and the limited therapeutic modalities for these carcinomas emphasize the importance of this finding. Targeted anti-GHRH therapy proved to be efficient in the treatment of nude mice transplanted with human TNBC xenografts. An unfortunate observation was the lack of diffuse GHRH-R expression in this molecular group. Whether this issue highlights a limited utilisabilityof a possible anti-GHRH-R treatment should be investigated in the future.

There was no association of GHRH-R expression and the nodal status of breast carcinomas.

Regarding metastatic breast cancer, axillary lymph node metastases of the GHRH-R expressing primary node positive tumors were evaluated. Although we noticed varying degree of GHRH-R staining decrease of the metastases compared to the primary carcinomas.Only a single case showed total loss of GHRH-R expression, which is an important observation if we consider that any future targeted therapylooks more promising if it could also help in advanced cases.

Due to the uniform GHRH-R expression noticed in cysts showing apocrine metaplasia, 31 cases of apocrine carcinoma were included in this study. With 10% cut-off, 97%demonstrated strong and diffuse positivity, whereas using 50% cut-off, 90% were found positive. As concerns the molecular types approached by IHC, somewhat more than half of apocrine carcinomas represent a subgroup of TNBCs and nearly half of them overexpress human epidermal growth factor receptor 2(HER2). A molecular apocrine type of breast cancer with increased androgen signaling has also been described. Their androgen-dependent signaling pathway could also suggest a specific treatment. Whether their homogeneous positivity for GHRH-R can be translated to a targeted therapy with GHRH-R antagonists, which are under development for clinical use requires further studies.

5.2. p40 EXPRESSION IN BASAL-LIKE BREAST CARCINOMAS AND p40 AS A MYOEPITHELIAL MARKER IN BREAST LESIONS

The identification of an outer MEC layer is a valuableclue in the differential diagnosis of breast lesions. A broadspectrum of different MEC markers is used (e.g. smooth muscle actin (SMA), smooth muscle myosin heavy chain (SMMHC), calponin, S100, CK5/6, S100, p63, and CD10).Due toits high sensitivity and even

superior specificity, p63 is preferred tocytoplasmic markers (SMA, calponin and SMMHC). Reduced expression of some markers (CD10, CK5/6 and SMMHC)in MEC associated withDCIS, AME and complex sclerosing lesions is a documentedphenomenon. This study specifically focused on these lesions,whichhave been reported to demonstrate an altered MECphenotype. Our results suggest that p63and p40 perform similarly in all these settings. In normalbreast tissue MECs are nicely highlighted by both antibodies,and when the expression of one is reduced in a pathologicalcondition, the other shows a similar reduction inexpression; focal losses of expression occurred in parallel.Although p40 has been reported to have superior specificitythan p63 as a squamous cell carcinoma marker inthe differential diagnosis of non-small cell lung cancer, it seems to perform similarly in breast lesions acknowledgedto show altered expression of MEC markers.It is, therefore, suggested that both antibodies can be usedinterchangeably for the demonstration of MEC. A recent study performing a TMA analysis of a largernumber of breast lesionsreached a similar conclusion.

The molecular subtype of breast cancer carries valuable information and can help to predict prognosis and determine the appropriate therapy. As long as determination of molecular subtypes based on gene expression profiling is not yet available in routine histopathology practice, surrogate IHC methods are extensively used. Using the IHC based method, BLBC is defined as an ER, PR and HER2 negative tumor expressing proteins usually found in basal/ myoepithelial cells. Although CK5 and EGFR are the most frequently used, other markers e.g. CK6, CK14, CK17, P-cadherin, CD117, nestin, p16 and p53 can also be used. As concerns the p53 tumor suppressor gene family, both p53 and p63 expression can be used as markers of basal phenotype. The anti-p53 antibody has a specificity of 80-85% and a sensitivity of 50-60%, whereas the detection of the p63 protein expression is reported to have a very high specificity (94%), but low sensitivity (14%).p40 was recently introduced as a commercially available antibody and was not previously tested in BLBC. CK5-expressing TNBCs seem to express p40 more frequently than p63. Whether this phenomenon is restricted or preferential in BLBCs expressing CK5 has not been examined, and is the subject of an ongoing investigation.

The presence of tumor cell positivity in NST carcinomas demonstrating an IHC staining profile mostly in keeping with a BLBC did not interfere with MEC detection but should be acknowledged.

5.3. CD10 EXPRESSION IN APOCRINE LESIONS OF THE BREAST

The fact that CD10 is a ubiquitous enzyme found onthe surface of many different normal cell types and pathologiclesions has a negative impact on its specificity andthus on its possible utility in routine histopathologicaldifferential diagnosis. Therefore, CD10 IHC reactionsshould be only used to answer specific differential diagnosticquestions in well-known circumstances.

Although breast epithelium rarely expressesCD10, positivity inmetastatic tumors cannot rule out the breast as primary.NSTcarcinomas and ILCs are rarely positive forCD10, but some subsets may be different in this respect:of 40 ER-positive tumors, none demonstratedCD10 positivity, whereas 12of 77 ER-negative carcinomas showed cytoplasmicor membranous staining. Apocrine carcinomas are also generally ER- and PR-negative.Some TNBCs expressbasal (i.e. MEC) markers and this feature has been suggested for the delineationof the BLBCsubgroup of breast cancers on IHC. Not surprisingly,some of these carcinomas may also express CD10, aMEC marker in a substantial number of cases.

CD10 positivity has been described in benign apocrine epithelium, but no data on CD10 expression in various other types of apocrine breast lesions have been available until now. Our results indicate that benign apocrine epithelium is typically positive for CD10 with a luminal staining pattern, although there are exceptions to the rule. Apocrine differentiation in malignant lesions seems to be associated with a partial or complete loss of this staining pattern, which is therefore rarer in in situ carcinomas and even rarer in invasive tumors, and cytoplasmic (aberrant) staining may also occur in this subset.

6. CONCLUSIONS

Our work demonstrates that the distribution of GHRH-R positivity among breast carcinomas is not restricted to histological type, differentiation grades or molecular subtypes. ILCs were found to express this marker more frequently than ductal/NST carcinomas. The finding of a relatively high proportion of positivity among carcinomas with casting-type microcalcification is of uncertain significance. Even though TNBCs showed GHRH-R positivity in a relatively low percentage of cases and tumor cells, with no cases showing positivity in more than half of the tumor cells, the unfavorable prognosis and the limited therapeutic modalities available for these patients highlight the importance of this finding.These results are further emphasized by the fact that targeted anti-GHRH-R therapy is proved to be efficient in the treatment of nude mice transplanted with human TNBC xenografts.The most remarkable finding of this study, we feel, is that apocrine carcinomas stain diffusely and strongly for GHRH-R. Whether our findings can be used for targeting breast carcinomas with GHRH antagonists is to be clarified in future studies.

The p40 protein seems to be similar to p63 as a MEC marker both in normal breast tissue and in lesions with observed alterations in the MEC immunophenotype. The presence of tumor cell positivity in NST carcinomas demonstrating an IHC staining profile mostly in keeping with a BLBC did not interfere with MEC detection but should be acknowledged, and the preference of p40 for highlighting this subset of carcinomas rather than other subtypes should be further investigated. Due to its usually focal staining pattern p40 is not an ideal IHC marker of BLBC.

CD10 positivity is luminal/membranous in most benign apocrine lesions, the staining being non-universal and sometimes focal. Analogous staining in apocrine malignancies seems rarer in DCIS and even rarer in invasive apocrine carcinomas, but atypical cytoplasmic positivity may also occur. CD10 is not an ideal MEC marker in apocrine lesions.When using CD10 IHCas a MEC marker or in the case of a carcinoma of unknown primary it should be important to know that benign and malignant apocrine lesions of the breast can also express CD10. The fact that CD10 is a ubiquitous enzyme found onthe surface of many different normal cell types and pathologiclesions has a negative impact on its specificity andthus on its possible utility in routine histopathologicaldifferential diagnosis. Therefore, CD10 IHC reactionsshould only be used to answer specific differential diagnosticquestions.

7. ACKNOWLEDGEMENTS

I wish to express special thanks to my supervisor **Professor Gábor Cserni** from the Department of Pathology, University of Szeged and Head of the Department of Pathology, Bács-Kiskun County Teaching Hospital in Kecskemét, for his support and scientific guidance of my work.

I could not be any more thankful to **Professor Béla Iványi,** Head of the Department of Pathology, University of Szeged, who provided more than excellent working conditions at the institute.

I am grateful to my tutors **Sándor Hamar, László Kaizer** and **András Vörös** who introduced me to the world of breast pathology and routine histopathology practice.

I greatly appreciate all the hard work provided by the staff members of the breast tumor board: **Professor Zsuzsanna Kahán**, **Professor György Lázár**, **Attila Paszt**, **Zsolt Simonka, Katalin Ormándi**, **Csilla Hoffmann**, **Máté Lázár**, **Gyöngyi Kelemen**, **Aliz Nikolényi**, **Erzsébet Valicsek**, **Ágnes Dobi**, **Zsófia Együd, Orsolya Rusz**, **Professor Gábor Cserni**, **Sándor Hamar**, **László Kaizer, András Vörös, Ágnes Báthori, Tamás Zombori** and the assistants (especially to **Beáta Balogh and Krisztián Daru)**, and photo technician (**Mihály Dezső**) of the Department of Pathology, University of Szeged that helped this dissertation to come into existence.

I would also like to express my gratitude for **all my other colleagues and coworkers** (especially to **Professor Éva Kemény and Professor László Tiszlavicz)** who are not involved in breast pathology but also helped me becoming a pathologist.

I also thank my **family and friends** for love, encouragement and support.

The work reported in this thesis was partially supported by grants **TÁMOP-4.2.2.A-11/1/KONV-2012-0035** and **KEP 2013, 2014 and 2015 of the University of Szeged**.