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Cilla Honkamäki

Characterization of a new, *in vivo* model of breast cancer lung metastasis

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This Bachelor's thesis was made in research group which operates in the Laboratory of Molecular Oncology, at the Biomedicum, University of Helsinki, Finland. Bachelor's thesis aim is to characterize of a new *in vivo* model of breast cancer lung metastasis. Research group has started their study recently and the study will continue further to cancer drugs.

Cancer is a worldwide and common fatal disease. Cancer has been studied over time and is an important subject of research today. The studies over time have led to the discovery of new medicines and therapies to cancer. The origin of cancer is the due of many factors. Breast cancer is heterogeneous disease and it is the most common cancer in women. Breast cancer can be aggressive and metastatic which is related with poor prognosis.

One of the important tyrosine kinase proteins in breast cancer is human epidermal growth factor receptor HER-2 and its overexpression is associated to cancer development. It acts as an activator in many signaling cascades and a part in cell growth, dividing, proliferation and cells repairing themselves. HER-2 is commonly used biomarker in the diagnosis of breast cancer, and it is also used in prognosis and treatment planning.

Cytokeratins are composed of keratin proteins and expressed in all epithelial cells. Those are intermediate filaments in eukaryote epithelial cells and thus are part of the cytoskeleton. Cytokeratins are typically expressed in different types of carcinomas and cancer cases. Anti-cytokeratins are specific markers for epithelial cell differentiation and are used as tools for the classification and identification of tumors.

Tumor samples were stained using two-step immunohistochemical staining with two different antibodies, HER-2 and Anti-Pan Cytokeratin. Immunohistochemical staining is an antibody-antigen method and positive result is shown as brown color.

The results are based on immunohistochemical staining's shown in the figures. The characterization has been implemented by describing the metastasis of tumors and their cell structure and comparing the two different antibody staining. I did the conclusions of the results, based on the theory of earlier studies.

Keywords	cancer, breast cancer, metastasis, tumor, HER-2, cytokeratin, IHC

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Tämä opinnäytetyö on toteutettu tutkimusryhmässä, joka toimii molekulaarisen onkologian laboratoriossa, Biomedicumissa, Helsingissä. Opinnäytetyöni tarkoituksena oli karakterisoida rintasyövän keuhkometastaasien uutta *in vivo*-mallia. Syöpätutkimuksiin kohdentunut tutkimusryhmä on aloittanut tämän tutkimuksen vähän aikaa sitten, ja he jatkavat tutkimusta syöpälääkkeisiin.

Syöpä on maailmanlaajuinen ja yleinen kuolemaan johtava sairaus. Syöpää on tutkittu ajan mittaan paljon, ja se on tärkeä tutkimuksen kohde. Aiemmin tehdyt tutkimukset ovat johtaneet uusien syöpälääkkeiden ja hoitomuotojen löytämiseen. Syöpä on monen tekijän aiheuttama sairaus. Rintasyöpä on heterogeeninen sairaus ja se on naisilla yleisin syöpä. Rintasyöpä voi olla aggressiivinen ja metastasoiva, jolloin myös syövän ennuste on huonompi.

Yksi rintasyövän tärkeistä tyrosiinikinaasiproteiineista on ihmisen epidermaalinen kasvutekijä reseptori 2 (HER-2) ja sen yli-ilmentyminen liittyy vahvasti syövän kehitykseen. Se toimii aktivaattorina monissa signalointikaskadeissa ja osana solujen kasvua, jakautumista, lisääntymistä ja solujen korjautumista. HER-2 on yleisesti käytetty biomarkkeri rintasyövän diagnosoinnissa, ja sitä käytetään myös syövän ennusteen teossa ja hoidon suunnittelussa.

Sytokeratiinit koostuvat keratiiniproteiineista, ilmenevät kaikissa epiteelisoluissa ja ne ovat osa solun tukirankaa. Sytokeratiinit ovat siis väli filamentteja eukaryoottien epiteelisoluissa. Sytokeratiinit ilmenevät tyypillisesti erityyppisissä karsinoomissa ja syöpätaudeissa. Sytokeratiinit ovat spesifisiä markkereita epiteelisolujen erilaistumiseen, ja niitä käytetään välineinä kasvainten luokittelussa ja tunnistamisessa.

Keuhkometastaasien karakterisointiin käytin immunohistokemiallista värjäystä kahdella erilaisella vasta-aineella, HER-2:lla ja Anti-Pan-Cytokeratiini:lla. Immunohistokemiallinen värjäys on vasta-aine-antigeenimenetelmä ja positiivinen tulos ilmenee ruskeana värinä.

Tulokset perustuvat immunohistokemiallisiin värjäyksiin. Karakterisointi on toteutettu kuvaamalla metastaasikasvaimia, niiden solurakennetta ja vertaamalla kahta käytettyä vasta-ainevärjäystä. Tein tulosten johtopäätökset aiemmin tehtyjen tutkimusten teoria pohjaan perustuen.

Avainsanat	syöpä, rintasyöpä, etäpesäke, HER-2, Sytokeratiinit, immunohistokemia

Contents

Abbreviations

ABS	Absolute alcohol
Apoptosis	Programmed cell death
CK	Cytokeratin
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGFR	Human epidermal growth factor receptor
ER	Estrogen receptor
EtOH	Ethyl alcohol
EU	European union
HER-1	Human epidermal growth factor receptor 1
HER-2	Human epidermal growth factor receptor 2
HER-3	Human epidermal growth factor receptor 3
HER-4	Human epidermal growth factor receptor 4
HRP	Horseradish peroxidase
IARC	International Agency for Research on Cancer
IHC	Immunohistochemistry
JIMT-1	Breast cancer cell line
p53	Tumor suppressor protein
PR	Progesterone receptor
TBS	Tris-buffer saline
TIFF	Tagged Image File Format
TP53	Coding gene for protein p53

1 Introduction

This bachelor's thesis was produced as a part of Mark Barok MD Ph.D. research project under his professional guidance. The group operates in the Laboratory of Molecular Oncology, at Biomedicum, University of Helsinki, Finland and aims to establish novel *in vivo* models of metastatic breast cancer and test anti-cancer drugs with the model. Here, I show the optimization results of immunohistochemical labelling by utilizing key markers used to investigate the morphology of breast cancer metastases in mouse lung tissue and characterize the metastases based on the results.

Cancer is a global disease and causes many deaths a year. Malignant, aggressive and metastatic cancer has the worst prognosis. Cancer and cancer treatments are commonly studied subject around the world. (World Cancer Research Fund. 2018.) Around 15–20 % of breast cancers are HER-2 positive. HER-2 positivity is related to poor prognosis and, higher incidence of mortality, and therefore plays a role in treatment planning. Cytokeratins are intermediate filaments in eukaryote epithelial cells and typically expressed in different types of carcinomas and cancer cases. Cytokeratins are used as markers in tumor diagnostics. HER-2 and cytokeratins are used to estimate the prognosis of breast cancer and the level of tumor aggressiveness. (Fletcher 2019; Karantza 2011; Mitri — Constantine — O'Regan 2012; Schweizer et. al. 2006.)

The aim of my thesis is to characterize of a new *in vivo* model of breast cancer's lung metastasis. During the study I used two different immunohistochemical staining antibodies, compared the results of the both staining and described the metastatic tumors in the lungs of immunocompromised mice. All the samples are from different immunocompromised mice injected with JIMT-1 carcinoma cancer cell line. I sliced sections from paraffine embedded tumor blocks by microtome. Results are shown by images and they are descripted and summarized below.

Immunohistochemistry is the main method used in this work. Immunohistochemical staining has been in use in diagnostics, planning treatment based on the cancer characteristics, determination of the primary tumor origin for metastatic cancer, tumor classification, identification of structures and as a disease prognostic biomarker. The staining's in are including two different antibodies, which are also typically used in part of cancer diagnostics. (Duraiyan — Govindarajan — Kaliyappan — Palanisamy 2012.)

Mice are strongly involved in research, which underlines the importance of ethics. Mice are the most commonly used mammals in cancer and cancer drug studies. The ethics is important when study involves living beings and there are laws in Finland that control and prescribe research involving animals. There are also EU directives in Europe that regulate the use of animals. (Government Decree on the Protection of Animals Used for Scientific or Educational Purposes 564/2013; European Parliament and the Council of the European Union. Directive 2010/63/EU; Perlman 2016.)

2 Theoretical starting points

2.1 Cancer

Cancer today is one of the most studied diseases worldwide and many different methods and variations have been used in cancer researches (Seyfried — Huysentruyt 2014). International Agency for Research on Cancer (IARC) points out in their global cancer data report (2018) that cancer is a global and very dangerous disease and over half of the cancer cases reported lead to death. Today the cancer incidence is related to the population aging and growth. The origin of cancer is the due of genetic changes and cancer is known to be caused by predisposing cells to substances possessing the ability to cause uncontrolled cell division, called carcinogens. Carcinogens include external factors as environment and lifestyle and internal factors as metabolism, age, and heredity. (Hanselmann — Welter 2016.)

For functioning properly, the cell must occupy its correct position and divide if necessary and die through programmed cell death (apoptosis) when it is required. Thus, proto-oncogenes are essential genes for normal cellular function and their protein products are involved in many cell growth-regulating functions such as cell cycle regulation, apoptosis, and differentiation. A proto-oncogene may become an oncogene due to a point mutation that results in a continuously active protein product or excessive amplification of the proto-oncogene resulting in overproduction of the protein, or translocation causing higher expression of the protein. This oncogene activation typically results in increased cell division, decreased cell differentiation, and inhibition of apoptosis. In addition to oncogene, one or more of the tumor suppressor genes must stop working properly, and for that it needs damages in both gene copies in the cell for uncontrolled proliferation. Tumor suppressor genes are the ones which in normal circumstances slow down cell division, con-

tribute to the DNA mistake repair process, or leads the cell to apoptosis (American Cancer Society 2014). An example of tumor suppression genes is TP53 which encodes the p53 protein, which in turn participates in cell cycle regulation by stopping the cell cycle if its DNA is damaged. Thus, if p53 is inactivated, the cell will continue to divide, even if the DNA is damaged, and the cell divides. The daughter cells formed by cell division do not have the correct number of genes, and they can harbor new mutations caused by DNA damage or its not-appropriate repair. Therefore, activation of oncogene and inhibition of the tumor suppressor genes leads to the survival of the cell instead of apoptosis. When the cell has these genetic defects, it is allowed to divide uncontrollably and create new daughter cells with the same abnormal behavior and abnormal structure, then cancer can occur. (Alberts et.al. 2004: 726-733; American Cancer Society 2014; Bray et. al. 2018; Chial 2008; World Cancer Research Fund 2018.)

Breast cancer is heterogeneous disease and the most diagnosed and common cancer in women. It causes many deaths and has been in 2018 the second most common cause of cancer deaths. Breasts contain luminal cells in the inner part and myoepithelial (basal) cells in the outer part. Histopathological classification in breast cancer cases are based on gene expressions profiling. Basic molecular subtypes of breast cancer are Luminal A, Luminal B, HER-2-enriched and basal-like. Each subtype has been shown to differ in incidence, prognosis, response to treatment, preferential metastatic organs, and recurrence or disease-free survival outcomes. Basal-like is a unique subtype among breast cancers and it is usually triple negative breast cancer, which means estrogen receptor (ER) -negative, progesterone receptor (PR) -negative, and HER2-negative. It has the worst prognosis and worse outcome than Luminal-A or Luminal-B subtype. However, Martin-Castillo et al. has shown in their study that there is basal-HER-2+ subtype which is associated with basal-like subtype. This basal-HER2+ subtype is positive for basal cytokeratins as well. (Cho 2016; Bray et. al. 2018; Martin-Castillo et. al. 2015.)

2.2 Metastasis

Once the cancer cell is formed it begins to differentiate and divide uncontrollably forming a primary tumor. A tumor is malignant when the cells invade the surrounding tissue. Due to this invasion, malignant cells may leave the primary tumor and be transported via the lymphatic or circulatory systems and might form a secondary tumor, called metastasis, that proliferates elsewhere in the body. Metastases are generally responsible for cancer deaths worldwide, and this high mortality rate is the main reason, why cancer studies are

so important. An example in metastatic malignant breast cancers the cancer cells invades surrounding tissue, and cells transport through the lymphatic system into the lymph node and then into the lungs thereby forming lung metastasis. Usually, metastatic cancer is associated with poor prognosis and a higher risk of mortality in cancer cases. However, the cells in the metastases are similar to the origin of the primary tumor. Thus, this means that if breast cancer metastasizes to the lungs, the secondary tumor consists of breast cancer cells, not abnormal lung cells. The tumor in the lungs is then called metastatic breast cancer, not lung cancer. (Alberts et.al. 2004: 726-733; Seyfried — Huysentruyt 2014; Martin — Ye — Sanders — Lane — Jiang 2013.)

2.3 The JIMT human cancer cell line and mice model

In a study Tanner et al. (2004) created a carcinoma cell line (JIMT-1) from pleural metastases from a 62-year-old breast cancer patient. The patient was clinically resistant to trastuzumab. The established cell line has the amplification of the HER-2 oncogene and it is resistant to trastuzumab both *in vitro* and *in vivo* mouse models, which provides a good model for example studies of trastuzumab resistance mechanisms. Consequently, the JIMT-1 is a human HER-2- positive breast cancer cell line which has been in use at earlier in the research group's studies. In this project cell culture was done in Biomedicum, Helsinki, in cell culture room by one of research team member. For this research, immunocompromised mice were injected intravenously with 100 000 JIMT-1 cells. JIMT-1 should be cytokeratin positive and Tanner. et al. has shown it has been positive for specific CK8, CK18, CK5 and CK14 cytokeratins.

Immunocompromised mice are commonly used models in cancer and cancer drug researches. Mice models can closely mimic, for example, human epithelial carcinogenesis, which provides ample scope for *in vivo* studies. Mouse models represent cellular and molecular changes in cancer and therefore play a crucial role in cancer studies and cancer medication studies. Despite, immunocompromised mice are used to study tumors, uncertainties may arise due to the inability to ensure tumor initiation and rate of development. Immune deficiency is one of the risk factors for cancer and immunocompromised mice how they express T cells, B cells, and macrophages, and NK cells. In this experiment we used nude mice which have no T cells but have B cells, and macrophages, and NK-cells. The advantage of using mice models is that it gives better opportunities to investigate many cancer-related biological process and also an effect of drugs in a living organism, which is much more complex than the *in vitro* experiments and closer to the

real situation of a patient with metastatic cancer. (Lei — Ren — Wang — Liang — Tang 2016.)

2.4 HER-2

The human epidermal growth factor receptor 2 (HER-2), is a tyrosine kinase and is a transmembrane protein on the cell surface. HER-2 is able of intracellular signalling and acts as an activator in many signalling cascades. It amplifies signals from other members of the epidermal growth factor receptor (EGFR) family (HER-1, HER-3 and HER-4). Additionally, activation of HER-2 also causes changes in gene expression mediated by transcription translation and protein stability. Due this activation, HER-2 acts part in cell growth, dividing, proliferation and cells repairing themselves. However, if HER-2 is abnormal and overexpressed in the cell membrane it causes an uncontrolled rate in cell growth, and proliferation. The EGFR family, including epidermal growth factor receptors HER-1, HER-2, HER-3 and HER-4, is one of the most characterized systems in breast cancer. HER-2 is overexpressed in 15–20 % of breast cancers and HER-2 overexpression has been shown to occur also in other cancers such as lung, gastric, oral and ovarian cancers. (Barok et.al. 2018; Fletcher 2019; Mitri et. al. 2012; Meric-Bernstam — Hung 2006.)

Breast cancer can have HER-2 gene amplification, and this can lead to a significant amount of HER-2 receptor expression at the tumor cell surface (Moasser 2011). HER2 positivity is one of five major biologically intrinsic subtypes in breast cancers. HER-2 positive cancer is a more aggressive disease, with a higher relapse rate and increased mortality. Trastuzumab is an anti-HER-2 monoclonal antibody drug used to treat HER2 positive breast cancer and gastric cancer, in combination with chemotherapy and it has been shown to be effective and improve the prognosis (Slamon et. al. 2001). Trastuzumab has been used since 1998 for the treatment of HER-2 positive metastatic breast cancer. (Mitri et. al. 2012; Meric-Bernstam — Hung 2006). In breast cancer diagnostics, HER-2 staining is part of tumor classification, which is based on morphology and molecular proliferations, and it also helps with prognosis and planning the treatment for cancer to avoid mortality. (Minot et. al. 2012)

2.5 Cytokeratins

Intermediate filaments composed form keratin proteins in eukaryote epithelial cells are known as cytokeratins (CK). They are expressed in all epithelial cells and are part of the cytoskeleton. Cytokeratin expression in the cell depends on the time of cell differentiation and the stage of development. There are two types of cytokeratins: acidic type I (9–23) and basic type II (1–8). Humans have 30 different genes which are encoding cytokeratins; 20 are epithelial genes and the rest 10 are specific for trogocytosis. Trogocytosis is also called immunological synapse and it refers to the cell-cell interaction of antigen presenting cells. In cells, cytokeratin forms a complex network in the cytoplasm, and it extends from the nucleus to the cells surface, supporting the nucleus and the cell itself. They play an important role in epithelial cells protected from mechanical and non-mechanical stress factors, and their important functions in cells include mobility, cell size, protein synthesis and membrane signalling. Phosphate exchanges take place in the cytokeratin network and mediate depolymerization, which in turn affects mitotic, post-mitotic division, cellular movement and differentiation. (Schweizer et. al. 2006; Gusterson — Heath — Ross — Stein 2005; Rechavi — Goldstein — Venitsky — Rotblat — Kloog 2007.)

Cytokeratins are typically expressed in different types of carcinomas and cancer cases. The expression or absence of cytokeratin in tumors and peripheral blood is prognostically significant in cancer cases and they have been widely and long in use as immunohistochemical markers in tumor diagnostics. Cytokeratins are involved in cancer cell invasion, metastasis and sensitivity to cancer treatment. Cytokeratins can be used to determine, for example, the prognosis of breast cancer and the tumor aggressiveness of cancer. Epidermal growth factor receptor and certain cytokeratin positivity are associated with high tumor levels and poor prognosis of cancer. The cytokeratins commonly expressed by breast cancer are CK5, CK6, CK7, CK14, CK17, CK18 and CK19. (Gusterson et. al. 2005; Karantza 2011.)

Anti-Pan Cytokeratin antibody diluent is specific for Type I (10, 14, 15, 16 & 19) and Type II (1, 3, 4, 5, 6 & 8) cytokeratins and it recognizes epitopes which are present in most human epithelial tissues. Anti-cytokeratins are specific markers for epithelial cell differentiation and are used as tools for the classification and identification of tumors. In this Anti-Pan Cytokeratin staining was used as positive control human skin tissue samples and as negative control mouse spleen tissue samples. This antibody reacts with human

cytokeratins. (Karantza: 2011. Appendix 2.) In cancer, cytokeratins are used as tumor markers in diagnostics because malignant tumors partially maintain specific keratin patterns associated with the original cells. The used cancer cell line was human breast cancer and healthy breast cells contain cytokeratins, so staining was used to experiment whether cytokeratin is also present in metastases. (Karantza 2011.)

2.6 Principle of Immunohistochemical staining

Immunohistochemical (IHC) methods are widely used in clinical diagnostics and research laboratories to determine the distribution of monoclonal and polyclonal antibodies in tissue sample slides. IHC staining has been increasingly used since the 1980s to detect the presence and location of specific proteins in a tissue section. The method has been used manually for a long time and nowadays automated alternatives are also available. The IHC method has applications in diagnostics, planning treatment, determination of the primary tumor origin for metastatic cancer, tumor classification, identification of structures and as a disease prognostic biomarker. (Luongo De Matos — Trufelli — Luongo De Matos — Da Silva Pinhal 2010.)

The staining is based on antibody-antigen binding and the principle of the technique is a specific antibody that will bind with its specific antigen to give an antibody-antigen complex. IHC methods can be either chromogenic or fluorescent. Chromogenic staining is visible by a light microscope and for fluorescence, the antibody is conjugated to a fluorophore that is detected by a fluorescence microscope. In the chromogenic staining the binding can be visualized by enzyme and in this work was used indirect detection. Indirect detection is a two-stepped process where the primary antibody binds the target antigen and enzyme-labelled secondary antibody binds to the primary antibody which is already bound to the antigen that can be visually detected. (Aptum Biologics; Duraiyan — Govindarajan — Kaliyappan — Palanisamy 2012.)

Tissues from sources need to be preserved quickly in order to prevent cellular protein and tissue degradation. Acetone, formalin or methanol may be used for fixation, depending on the target antigen. Formalin is a covalent crosslinking reagent that is commonly used as a fixative, and the attachment time depends on the degree of fixation desired. Tissues fixed in formalin are typically embedded in paraffin wax. Fixation keeps tissue shape, cell structure sharp and prevent endogenic and exogenic enzyme activity. Tissue sections need to be attached to glass slides that are suitable for immunohistochemical

staining. Methods used to determine the antigens in the tissue with the employment of specific antibodies that can be visualized through staining. (Luongo De Matos et. al. 2010.)

With formalin fixation, antigen epitope exposal is essential to allow the antibodies to bind. Usually, formalin fixation generates methylene bridges which are cross-linking proteins and thus mask the target epitope. The commonly used heat-induced antigen retrieval breaks the methylene bridges and exposes the epitope thereby allowing antibodies to bind. Chromogenic detection is based on antibodies conjugated to enzymes, such as commonly used horseradish peroxidase (HRP), to generate a color-producing reaction. When HRP is used, the activation of endogenous enzymes should be prevented to avoid the formation of nonspecific binding. Antibodies are diluted in an antibody buffer that helps stabilize the antibody and when the antibody is incubated it can bind to the target antigen in the sample. Antibody buffer can decrease non-specific binding. HRP binds to the antigen-antibody complex forming an antigen-antibody-HRP complex which reacts with DAB substrate when it is incubated correctly and forms the brown colour. Diaminobenzidine (DAB) is a chromogen for HRP and it is forming brown color which is insoluble in alcohol or water. Counterstaining with Hematoxylin is performed after antigen staining to create contrast and thus facilitate the visibility of the antigen and distinguish the staining result from the rest of the tissue. Hematoxylin gives a blue coloured counterstaining result to negative tissue. (Abcam. IHC-Paraffine; Luongo De Matos et. al. 2010; Paulsen — Dimke – Frische 2015.)

2.7 Cross-reaction in immunohistochemical staining

Cross-reactivity refers to the binding of a secondary antibody to a non-target antigen. The secondary or primary antibody can show an affinity for identical or similar epitopes on non-target antigens. A high primary antibody concentration will increase these interactions between the primary antibody and non-target epitopes and increase nonspecific binding and background staining. Polyclonal antibodies are more likely to cross-react and generate non-specific signal than monoclonal antibodies. Anti-Pan Cytokeratin is a monoclonal antibody so that cannot explain the cross-reaction. Cross-reactivity of species is also possible. The antigen-binding site of an antigen then recognizes immunoglobulin from one species, and it may also detect a homologous epitope in the immunoglobulin of another species. However, the solution for cross-rection is to reduce the con-

centration of the antibody. Another consideration that has been shown is that the antibody diluent should contain sodium chloride because it helps reduce ionic interactions and normal antibody diluent (ImmunoLogic) is containing it in this case. (Abcam. Immunohistochemistry; ThermoFisher. IHC troubleshoot guide.)

3 The study aims and purpose

The aim of this study is to establish and characterize a human, HER2-positive breast cancer lung metastasis model in immunocompromised mice. Characterization is based on images obtained by immunohistochemical staining. Later, the research group intends to use this model for studying the effect of different drugs in this model. Immunocompromised nude mice have been inoculated intravenously with HER2-positive human breast cancer cells (JIMT-1) to generate metastasis in the lung. The research includes an immunohistochemical comparison of the metastasis to the primary tumor and to the parental cells growing in the cell culture laboratory. This bachelor's thesis contains information on the cellular structure of human breast cancer cells and determines the role of certain proteins and receptors in the cells and cell membranes. The research group intends to establish cell lines from the lung metastases.

Leading research task of the thesis:

- Characterizing of a new *in vivo* model of breast cancer lung metastasis using immunohistochemical staining methods.

4 Methods and throughput

4.1 Tumor samples and preparation of slides

The JIMT-1 cells were cultured in a cell culture laboratory and injected into immunocompromised nude mice. The mice were sacrificed 17 weeks after the injection, and then samples from the lungs were collected and fixated. Mice sacrifices followed ethical principles, and all those who work with them have received separate training in animal-related researches. All the tumor samples are from different mice and samples were fixated in 4 % formalin at least 4 hours but not longer than 24 hours, and then processed with the automated tissue processing device. All the tissues were processed with automated

tissue processing device, operated in a separated laboratory in Biomedicum. After that tissue samples were embedded with paraffin wax forming paraffine blocks. Other members of the research group were doing tissue sample fixation, processing, and paraffin embedding. All the samples which are in use are from immunocompromised mice. Tumor samples were collected from mice and fixated correctly.

Slides were written by using a Primera Signature Slide Printer. The slides were placed in the printer, and a computer program is used to write the desired information on the frosted ends of the slides. Slides contained the information of sample tissue, identification information of the sample, information of which staining and its dilution, used incubation time, date when the sections and staining were made, and slide printer also added 2D bar codes to slides. Before staining I made sections of the paraffin-embedded tumor sample blocks with a Leica RM2255 automated microtome. I used the fully automated microtome manually. In order to get optimal sections, the blocks need to be cooled before slicing them. The cooling helps with making the sections and from cooled blocks, the sections are whole and smooth. I sliced 3–4 µm thin sections from the blocks and first I moved the slides to cold water with using small brushes and then I took the section to IHC method accepted glass slide and dipped it into hot water (55 °C) where the section stretches and it can be placed to the slide smoothly and directly.

4.2 Immunohistochemistry (IHC)

In this process, immunohistochemically methods contain two different antigens. Both IHC staining is using the same leading base method. I was using the Abcam IHC-Paraffine protocol to support the staining instructions from the lab. To prevent any interferences in staining results, paraffine needs to be removed carefully. Paraffin removal from the slides was accomplished as follows: slides in the xylene for 10 minutes then another vessel of xylene for 5 minutes and last xylene and absolute alcohol (ABS) dilution 50:50 for 3 minutes. Then begins xylene removing and rehydrate phase within decrease alcohol series where the slides will be about 10 dips of each vessel, it includes two ABS vessels, two 96 % ethyl alcohol (EtOH) vessels, one 70 % EtOH and one 50 % EtOH vessels and the phase will end in Aqua vessel. The rehydrate phase is important because IHC staining is happening in hydrous media and is removing hydration from the sample (Thermo Fisher. Antigen retrieval).

Hydrogen peroxide processing lasted 30 minutes, and hydrogen peroxide dilution was made with 5 ml 35 % H_2O_2 and 200 ml Aqua. Hydrogen peroxidase inactivates endogenous peroxidase which will prevent the non-specific background staining (IHCWORLD Peroxidase). Hydrogen peroxide dilution needs to be washed away with aqua after its 30 minutes incubation.

The antigen detection was implemented setting the slides to sodium citrate buffer pH 6 and running slides with Retriever the minimum 2 hours' time. Retriever heat up the slides in sodium citrate buffer and antigen is revealed within it and an antibody can access to the target. The slides must let cool down after Retriever handling and the sides are moved to slide holders under the aqua. Washing with Tris-buffered saline (TBS) solution is needed because the aqua should be washed away and to same time let you know the liquid will flow at the same speed within each slide holder. If the liquid passes through too fast when the antigen will be added to it, it's not going to fix in the slide even if the incubation time will be right. TBS washing is done two times and each time the minimum effect time is 5 minutes and notice liquid must be flown past the slide holder before the next wash or next phase.

In this phase, the specific antibody will be mixed to normal antibody diluent and mix were added to the slides. There are two different antibody diluents in use, 1:100 anti-HER-2 and 0,1 µg/ml Anti-Pan Cytokeratin. I used 30 minutes of incubation time within all implemented HER-2 staining. At first, I tried once overnight incubation, but the results were the same within 30 minutes incubation and overnight incubation, so I ended up doing the staining with 30 minutes incubation. The Anti-Pan Cytokeratin incubation time was overnight, so I started incubation right before I left from work and continued at the next morning. After incubation time there is needed to do TBS washing, also two times and within 5 minutes effect time for each.

After antibody incubation and TBS washing, it was time to add WellMed Orion, detection system to the slides, 100 µl for each and it needs 30 minutes incubation time. HRP Detection system detects primary antibody bound to an antigen in mice tissue sections and eliminates the backgrounds by blocking protein-protein interactions in IHC staining and stabilize the antibody and this way it ensures a better staining result. After the peroxidase detection system, it is time to do TBS to wash away the detection system from the slides.

ImmPACT DAB is the name of the product which is produced by Vector (2019) and the dilution was 1 ml ImmPACT DAB-diluent and 1 drop of DAB and it produces in the samples a brown color by reacting with HRP. The incubation time which I used was 5 minutes. Incubation time was in use at the research group and it is important to be the same at all staining's. ImmPACT DAB was washed away with TBS washing diluent 5 minutes and two times and before the next phase, I moved the slides to the aqua vessel. DAB is a carcinogen so it must be disposed of within dangerous garbage protocol. When I was handling this product, I used nitrile gloves and a lab coat to protect against skin contact.

For counterstaining, Mauer's hematoxylin was used in these two IHC staining methods. Slides were in Mayer's hematoxylin precise 1 minute. If the slides are in dye too long, it could give over the stained outcome and it will be amiss to the staining result. Straight after Mayer's hematoxylin, the slides were under running water 10 minutes and that washes away all the extra color. After Mayer's hematoxylin and running water the slides were handled with rising alcohol series again 10 dips for each. Series contains 50 % EtOH vessel, 70 % EtOH vessel, two 96 % EtOH vessels, two ABS vessels, 50:50 ABS and xylene dilution vessel for three minutes, and two xylene vessels for five minutes and 10 minutes. When the slides are in the last xylene vessel, I transferred it to another fume hood with a lid and I lifted the slides onto the blotting paper. Then the slides were covered using the three little drops of the glue on the cover glass and sliding it over the slide. After cluing the cover glasses to the slides, I left them to dry at least one day.

The dyed slides were pictured by using SlideStrider (SK-100) automated microscope scanner. An automatic microscope scanner was in use during the whole working. The slides were placed to the rack and the automatic microscope scanned the slides on its own. Automatic scanning took a lot of time and usually I started the microscope to scan the slides when I left work, so they were ready when I returned in the morning. I used the Fuji ImageJ program to process and cut the images with my own. This program was in use because that was a free and easy use program that could process (TIFF) tagged file format images.

The primary antibody may lose affinity for the target antigen due to long-term storage or freeze and thaw cycles. Also, changes in pH could cause this loss of affinity and that is why antibody diluent should always be ensured pH 7–8 which is optimum to antibody binding. Both antibodies were separated into small aliquots in sterile tubes and stored in

a freezer. That helped to avoid freeze and thaw cycles because whenever I made staining, I took only one small tube of antibody out of the freezer and made the right concentration mixture. I used universal IHC-blocking diluent, pH 7.3 which is optimal to antibody binding. I used control slides every time I stained samples. For the immunohistochemical staining of HER-2, we used an anti-human HER-2 specific rabbit IgG as a primary antibody. Human skin was used for negative control and OE19 human HER-2 positive gastric cancer xenograft was used as a positive control. Anti-Pan Cytokeratin staining we used human skin tissue as positive control and mice spleen as a negative control.

4.3 Optimizing Anti-Pan Cytokeratin staining

Anti-Pan Cytokeratin staining was introduced during my work and I optimized the staining with this antibody before staining the tissue sample slides. Anti-Pan Cytokeratin staining optimizing started from the incubation time. Concentration optimizing started from analyzing the 0,3 µg/ml and 0,5 µg/ml staining outcome because manufacturers product datasheet recommends 0,25 µg/ml–0,5 µg/ml concentrations (Abcam. Anti-Pan Cytokeratin Antibody; Appendix. 2). The first staining was done within concentrations 0,3 µg/ml and 0,5 µg/ml together with 1-hour and overnight incubations. Samples were the same with both concentrations and incubations, so the difference was easier to see. Overnight incubation was optimized for Anti-Pan Cytokeratin staining because from the staining results it is easy to see that overnight incubation is better and smoother than 1-hour incubation results. After first staining, it's sure that 0,5 µg/ml is too high concentration because the samples were strongly discolored so it was needed to find the perfect concentration for staining. When the staining result is over stained it's harder to differentiate the cells and tissue fragments. However, the best option was to do smaller concentrations than 0,3 µg/ml because within 0,3 µg/ml concentration stained sample slides were strongly stained too. The next staining was with 0,1 µg/ml, 0,2 µg/ml and 0,3 µg/ml concentrations and the samples which I stained were the same as in the first try. The 0,3 µg/ml was taken along to the lower staining to give a reference to lower concentrations.

5 Results

5.1 Optimizing

The results of the Anti-Pan Cytokeratin staining optimization are reported based on the figure below. I will tell more about the results and how the right staining concentration

was reached. Doing the optimizing of Anti-Pan Cytokeratin, it was shown clearly that within high concentration there were more cross-reaction than within a lower concentration.

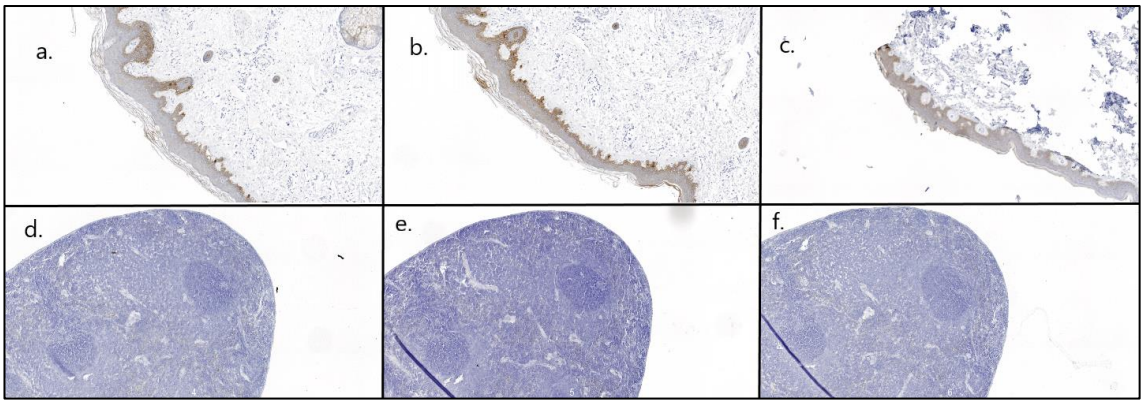

Figure 1. Positive human skin controls and negative mice spleen controls for Anti-Pan Cytokeratin staining, with different diluent ratio concentrations.

In figure 1 first row images are positive human skin controls with diluent ratio concentrations (a.) 0,1 µg/ml (b.) 0,2 µg/ml (c.) 0,3 µg/ml and second row are negative mice spleen controls and diluent ratio concentrations (d.) 0,1 µg/ml (e.) 0,2 µg/ml (f.) 0,3 µg/ml. The outcome was to optimize the Anti-Pan Cytokeratin staining to the 0,1 µg/ml concentration because of the staining results of slides that were stained within 0,1 µg/ml concentration were good, smooth and stained enough. Within 0,2 µg/ml concentration the staining result was over okay but it was giving some false positive epitopes in mice tissue and within 0,3 µg/ml the dying was overdyed and mixed so in the negative control there was a little positive brown dyed spot's in it. Anti-Pan Cytokeratin mixture was showing false positive epitopes in mice tissue, even if it should not. Concentration 0,1 µg/ml was the best because it was not giving false positive epitopes in mice tissue and the staining result was good and not over dyed (figure 1).

We optimized the Anti-Pan Cytokeratin staining because the research group had not previously used this staining. Optimization is important to achieve the best possible staining result and thus all future staining can be performed at the same concentration, which increases results reliability. The optimization was successful and did not require many staining times to complete.

5.2 Controls

Here is shown only the controls I used to stain the samples shown in the results. The controls are shown in the figures and opened in the text more below. Controls are important to achieve reliable results.

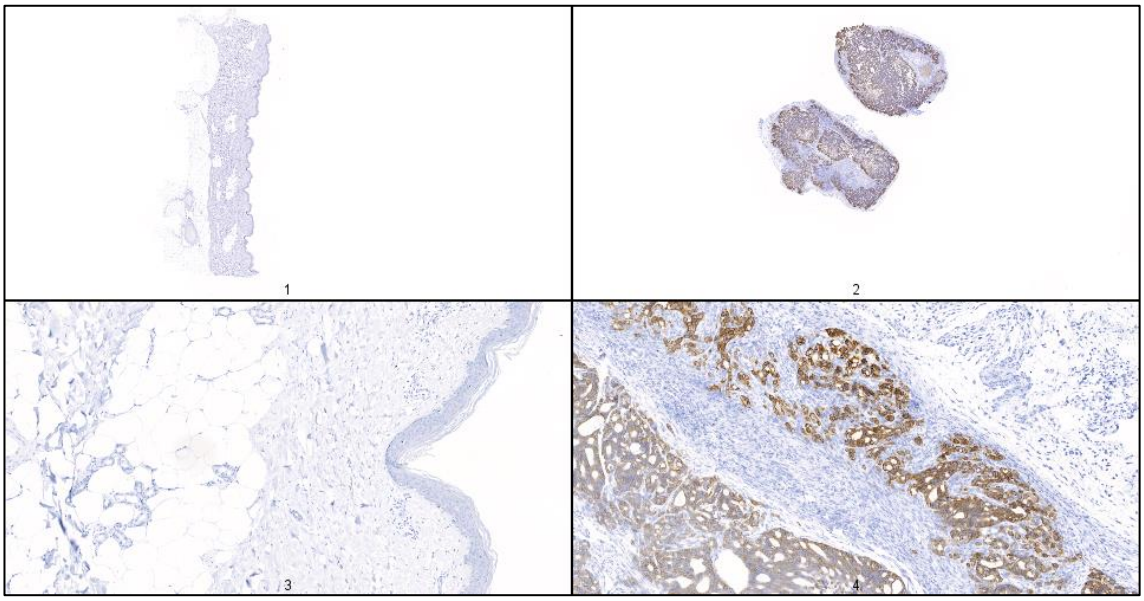

Figure 2. Control samples from HER-2 staining. 1 and 3 are negative controls (human skin), and 2 and 4 positive controls (HER-2 positive gastric cancer xenograft).

Negative control is human skin tissue and positive control is 96LuOE19 sample which were found to be a good control before I started working. 1 and 2 were controls are from staining tissue samples 218 JIMT and M1 JIMT. Those images are not zoomed but result color is visible from further too. In the image (1) human skin tissue is completely blue colored which means it is a negative control. Image 3 and 4 were control samples for tissue samples 235 JIMT, 236/1 JIMT, 236/2B JIMT and 234. In human skin tissue there is not any HER-2 and that is why it is good choice to negative control sample. 96LuOE19 positive sample is strongly positive and the staining result is smooth. In any of these control samples there was not any dye clumps nor cross reaction visible. (Figure 2.)

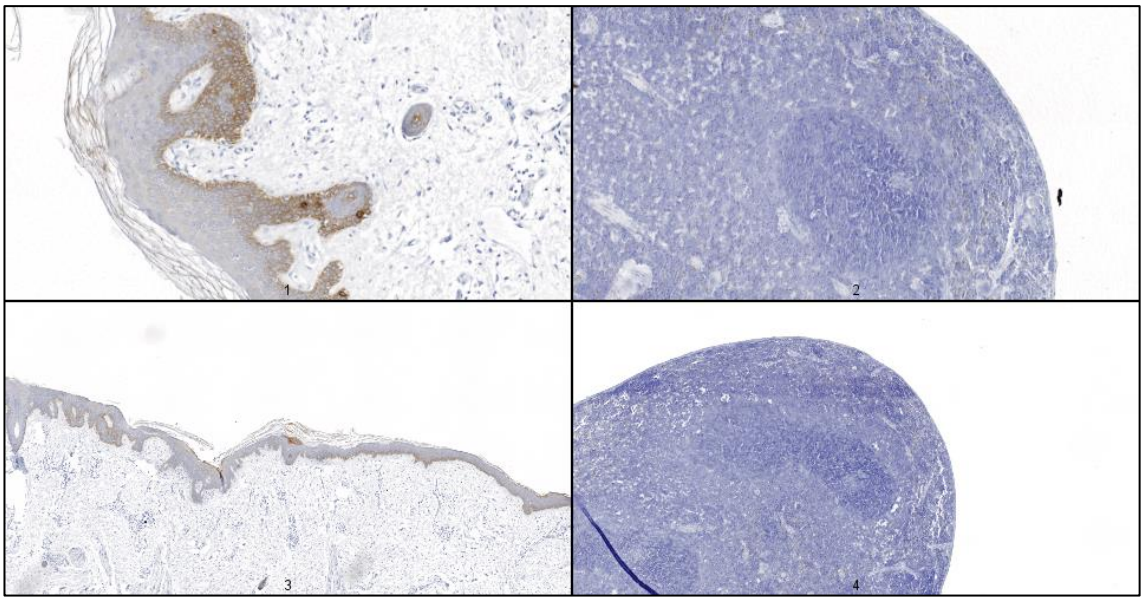

Figure 3. Control samples from Anti-Pan Cytokeratin staining.

I made all Anti-Pan Cytokeratin staining's with 0,1 µg/ml concentration and overnight incubation. In this staining human skin tissue sample and mice spleen tissue sample are used as positive and negative controls, respectively. In Figure 3 images 1 and 2 are controls from staining 218 JIMT, 234 JIMT and M1 JIMT samples. Images 3 and 4 served as a control samples from staining 235 JIMT, 236/1 JIMT and 236/2B JIMT samples. It is shown in Figure 3 that image 3 is slightly lighter in staining result than image 1. Staining results are visibly smooth and there were no markable dye clumps nor patch's shown in control samples.

5.3 Descript of tumors and comparing the staining's

JIMT-1 human breast cancer cell line was included in this work. The JIMT-1 cell line is a human breast cancer cell line, which is positive for HER-2 (Tanner. Et. al. 2004). All the used samples were mice lung tissue samples; the mice had been previously inoculated intravenously with the human JIMT-1 breast cancer cells. The mice lung tissue has negative staining result for HER-2 antigen as the antigen used has specific reactivity for human origin HER-2. Consequently, if we detect HER-2-positive cells in the mouse lungs, those should be human JIMT-1 breast cancer cells that metastasized the mouse lung.

I compared the results of two different staining and I also describe the morphology of metastasis tumors based on figures. I used two IHC methods and as the table 1 shows

there were two positive samples 218 TUMOR LUC and M1 JIMT LUC within both antigens and one which was negative within both 235 1/L JIMT. Two samples were positive within one antigen staining and negative within other 236 1/L JIMT and 236 2/B JIMT.

Table 1. This table shows all the used sample blocks and them IHC staining results.

Sample Block	Tissue	HER-2	Anti-Pan Cytokeratin
218 TUMOR LUC	LUNGS	pos. (+)	pos. (+)
M1 JIMT LUC	LUNGS	pos. (+)	pos. (+)
236 1/L JIMT	LUNGS	pos. (+)	neg. (-)
236 2/B JIMT	LUNGS	neg. (-)	pos. (+)
235 1/L JIMT	LUNGS	neg. (-)	neg. (-)
234/2 JIMT	LUNGS	pos. (+)	neg. (-)

The results are shown by the images which I took from stained slides. Characterizing of lung metastasis and comparing these two different staining results is based on the images below. The controls of the all done IHC staining's was shown and processed in the methods chapter in figure 1 and figure 2. In both staining's brown color means positive and blue color negative for antibody.

5.3.1 218 TUMOR LUC

The results of Sample 218 JIMT, HER-2, and Anti-Pan Cytokeratin staining are shown in the figures below. I have described the results based on the images and compared the two different staining results.

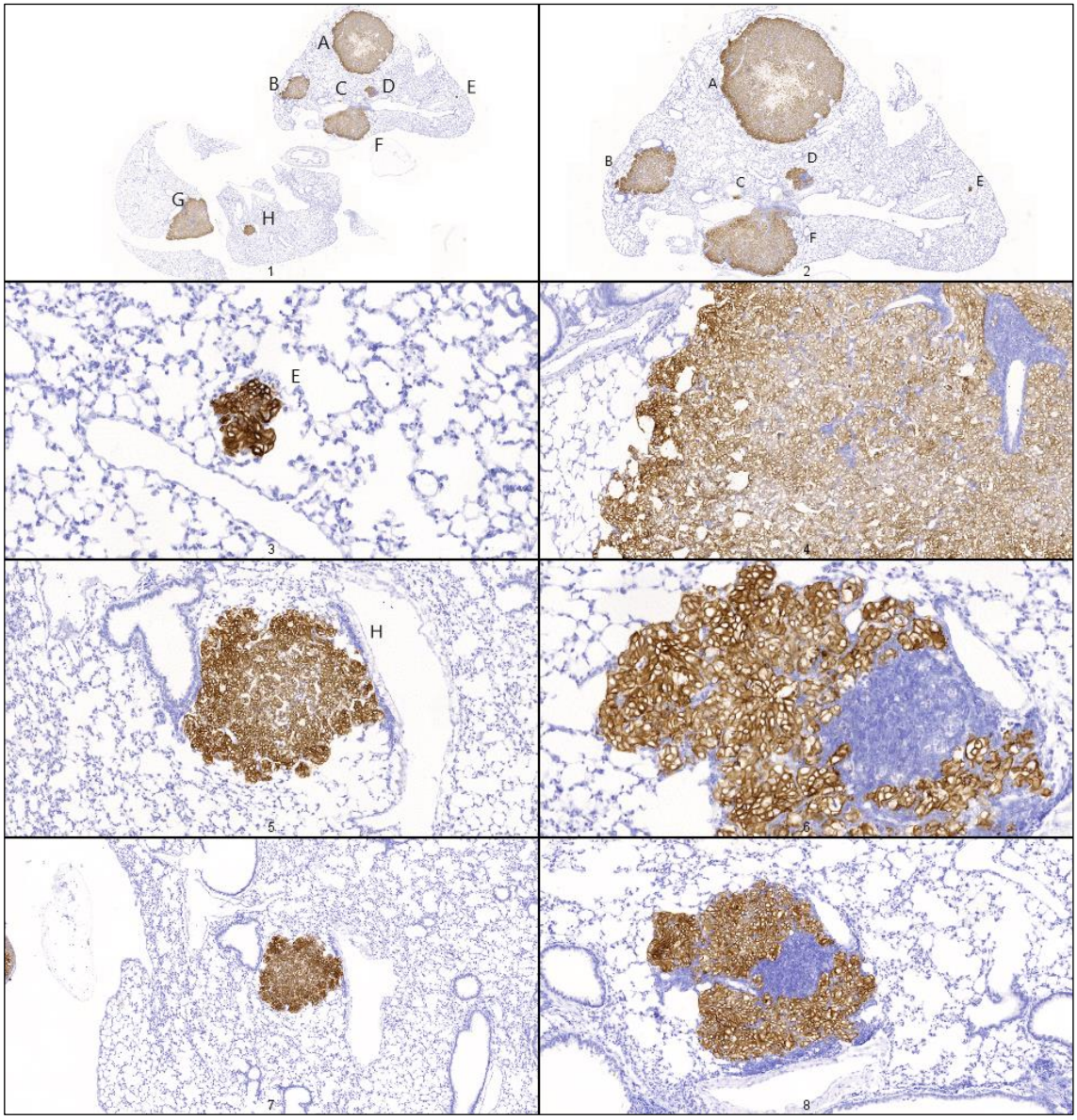

Figure 4. Sample 218 TUMOR LUC. HER-2 staining. Labeling A-H is naming the different metastases. 2-8 are zoomed images from different metastases.

As shown in figure 4 all the metastases have sharp boundaries and there are many different sized metastases in sample 218 TUMOR LUC. The color difference is clear in all images because cancer tissue is strongly positive for HER-2, and negative resulted tissue is well stained also. First and second images are from far and those are more like an overview of the whole sample and other images 3–8 are zoomed images. In this sample JIMT 218 there are eight different sized metastases which can be illustrated. The smallest one (E) is zoomed in image 3. There were two micro metastases (C) and (E), two small metastases (D) and (H), three medium-sized (B), (F) and (G) and one bigger

metastasis(A). The sample 218 TUMOR LUC is clearly strong positive for HER-2 protein in figure 4.

Metastases (C) and (E) were mini sized I assume they are under 100 cells sized. In both, the cells are densely organized, and the staining result is strong as shown example in figure 4 image 3. (D) and (H) metastases were small sized, and the cells are slightly more widespread than in the mini sized metastases, but still characteristic dense and abundant cell dividing is visible in images 5–8. In tumor (D), which is zoomed in images 6 and 8, there is visibly a negative site in metastasis and around it the tumor is positively stained. The negative site is also dense from cell organization and clearly associated with positive sites in metastasis and the it is clearly a part of the metastasis structure. The cell size of all metastases is similar, and the metastatic cells are larger than the lung tissue around them. Cell size have variation and it is visible in image 6. (Figure 4.)

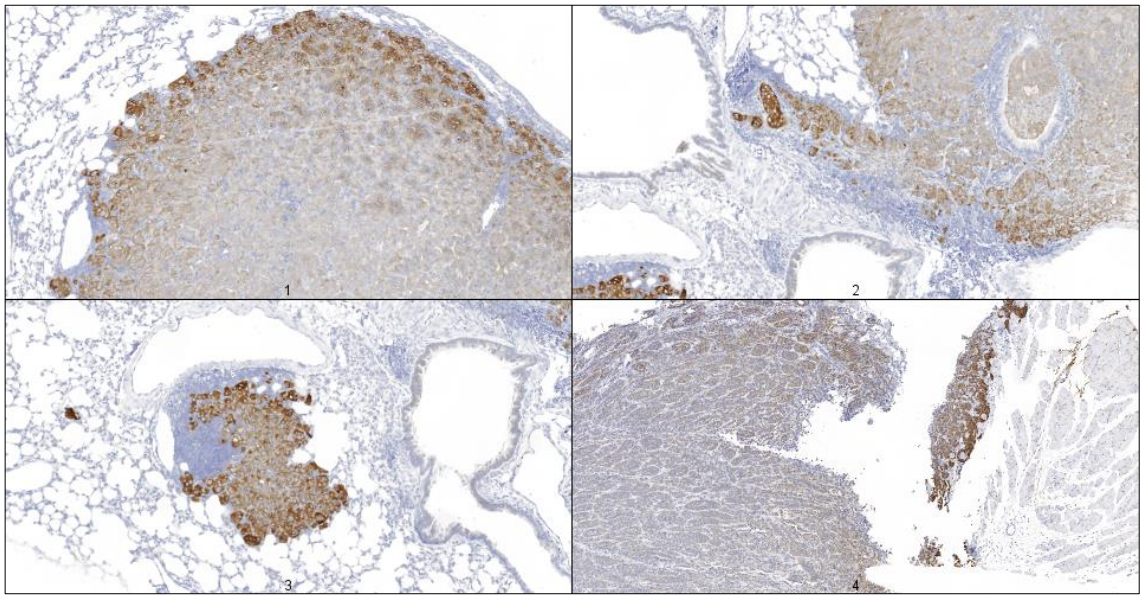

Figure 5. Sample 218 TUMOR LUC with Anti-Pan Cytokeratin staining. Images are zoomed from different metastases.

As figure 5 shows the images are zoomed of different metastases. Sample was at this point almost sliced to the end, so it is a bit split. Sample 218 JIMT is obviously positive for Anti-Pan Cytokeratin. The staining result of this Anti-Pan Cytokeratin staining is not as strong as HER-2 staining result. The metastases lining is sharp and clean as in the other staining, but the color is more faded. Smaller metastases as example in image 3 are more strongly dyed than the bigger metastases and in the bigger metastases the

edges are also more strongly stained than centers. In figure 5 image 1 Is zoomed image from the biggest metastasis. Image 2 is the same as metastasis (F) in figure 4.

In figure 5, image 3 there is sharp border between negative site and positive site of that tumor and positive cells at the borderline are clearly distinguished. In figure 5, image 2 metastasis is shown also cytokeratin negative area in it. The negativity is in edge of the metastasis and small part in center of it, this is the same one as in figure 4, (F) metastasis. It is visible that the negative site is part of tumors structure because of the organizing of the cells appears to be similar.

Image 3 in figure 5 is shown the metastasis (D) from figure 4. and both staining's for the sample 218 TUMOR LUC have negatively stained site in the tumor, and the site is located to the same place. The two images of the tumor are inverted relative to each other which may be a bit confusing, but if you take a closer look at the structure of the tumor then you may notice that it is at the same point.218 TUMOR LUC sample had the best staining result in both staining's. There was sharp lining between healthy and metastasis cancer tissue. This sample has eight metastases and indicates that the cancer has metastasized well to the lungs as desired.

5.3.2 M1 JIMT LUC

Below are the results of sample M1 JIMT LUC with HER-2 and Anti-Pan Cytokeratin staining. The description and presentation of the results are based on the figures and I have compared the results of two different staining.

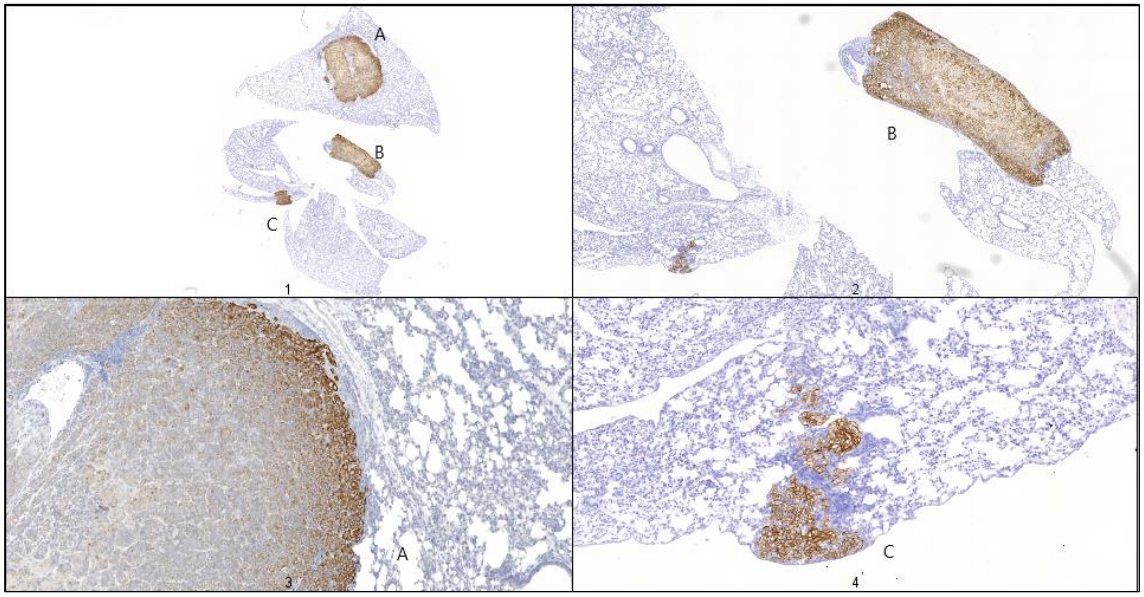

Figure 6. Sample M1 JIMT LUC. HER-2 staining. 1. Whole sample slide is shown. 2. Zoomed image of second biggest metastasis. 3. Zoomed image from biggest metastasis in the sample. 4. Zoomed image from the smallest metastasis.

In sample M1 JIMT LUC there were three different sized metastases in the mice lungs. Metastases are shown a variation cell size. There were one big (A), one medium sized (B) and one small (C) metastasis. The staining result is positive, well stained and bordering of the metastases are sharp in this sample. Positive, brown colored metastases are clearly distinguished from negative, blue colored lung tissue. As seen in figure 6, lung tissues cellular structure is more loose connective tissue than metastases, where tissue is tight connective. Medium sized metastasis (B) in image 2 is sharp bordered and having a tight cell structure and it is the densest of the metastases.

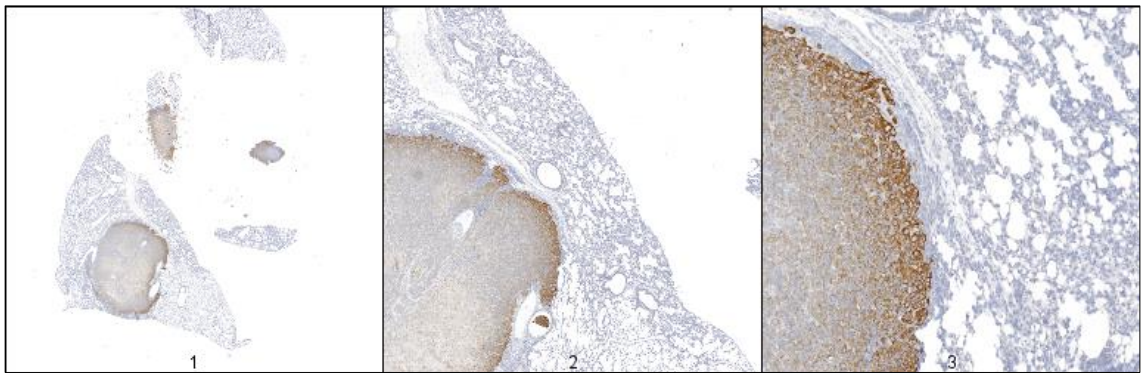

Figure 7. Sample M1 JIMT LUC. Anti-Pan Cytokeratin staining.

Sample M1 JIMT LUC is positive for Anti-Pan Cytokeratin staining. In figure 7 the zoomed image 3 of metastasis shows that cancer cells and healthy tissues lining is clear and sharp lining. The metastases are similar structure in this figure 7 than in HER-2 staining of the same sample in Figure 6 and all the same metastases are stained positive. Staining result is good compared to controls and based on staining of positive metastases and negative lung tissue.

Sample M1 JIMT was also strongly positive for both staining's and as sample 218 JIMT the both staining's metastases were visibly stood out well. All the metastases were well stained within HER-2 and Anti-Pan Cytokeratin staining. As the images showed there were exact boundary between metastases and healthy tissue in sample M1 JIMT with HER-2 staining and as well as with Anti-Pan Cytokeratin staining. In both samples, the cells of the metastases are clearly larger than the cells of the lung tissue and in metastasis cell size have variation.

5.3.3 236 2/B JIMT

HER-2 and Anti-Pan Cytokeratin staining results of sample 236 2/B JIMT are described below and shown by figures.

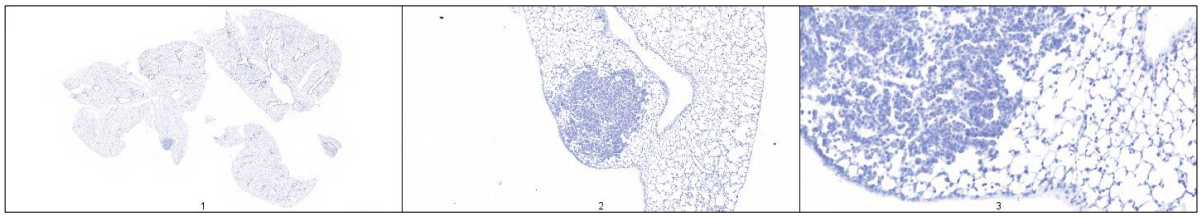

Figure 8. Sample 236 2/B JIMT. HER-2 staining.

Result of Sample 236 2/B JIMT HER-2 staining is negative because the staining result is completely blue. There is clearly shown metastasis in Figure 8, image 1 is overview, image 2 is zoomed in metastasis and image 3 is zoomed more closely. The cellular structure of this metastasis is clearly denser, and the cells are larger than elsewhere in the sample. The cells are grouped together which is characteristic of cancerous tumors, but it is noticeable that cell size variation is not that markable.

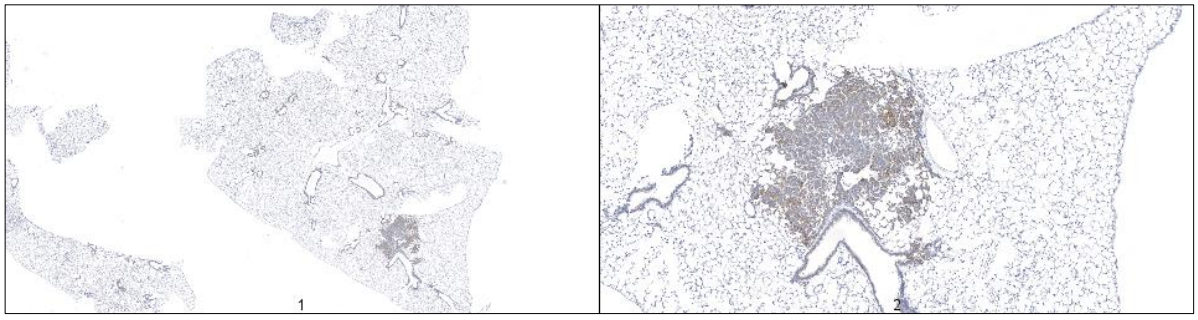

Figure 9. Sample 236 2/B JIMT. Anti-Pan Cytokeratin staining.

In Anti-Pan Cytokeratin staining samples 236 2/B result is positive. The staining result is not strongly positive but as image 2 in figure 9 shows there is brown color in the tumor and that means that there is some cytokeratins in the structure. The metastasis cell size is bigger than elsewhere in the lung tissue and the cells are more densely organized.

In sample 236 2/B JIMT is noticeable that cell size variation is not that markable. Both staining's are supporting that fact. Compared to samples 218 TUMOR LUC and M1 JIMT LUC the variation of cell size is much less in sample 236 2/B JIMT. It is also HER-2 negative which could support the fact that HER-2 expression in a cell's surface increases cells growth.

5.3.4 236 1/L JIMT

The staining result of sample 236 1/L JIMT Anti-Pan cytokeratin is shown below.

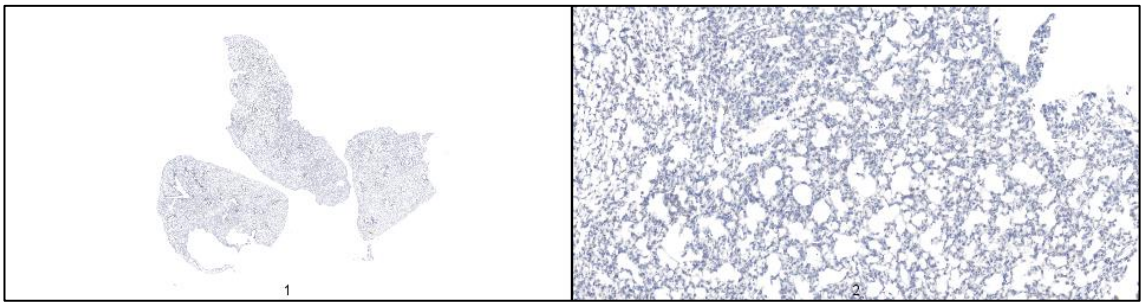

Figure 10. 236 1/L JIMT. Anti-Pan Cytokeratin staining.

Figure 10 is shown sample 236 1/L JIMT Anti-Pan cytokeratin staining and the result is negative. The image 1 is overall and image 2 is zoomed which is shown better the staining result and cellular structure is characteristic to lung tissue. It is blue and it means negative result and that there is none of the cytokeratins.

5.3.5 235 JIMT

Sample 235 JIMT was negative in, HER-2 and Anti-Pan Cytokeratin staining. I have shown figures of both staining results and describe them in greater detail below.

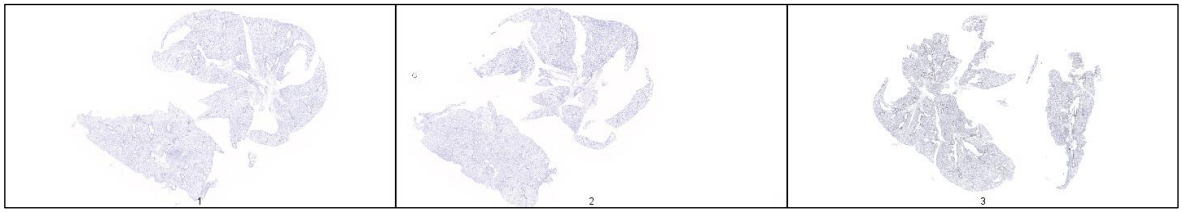

Figure 11. Sample 235 JIMT. Images 1. and 2. Are with HER-2 staining. Image 3. Is with Anti-Pan Cytokeratin staining.

In figure 11 Sample 235 JIMT the first two images are from HER-2 staining and image3 is from Anti-Pan Cytokeratin staining. As figure 11 shows the result of HER-2 staining was light and it is entirely blue so that means the result is negative. Images 1 and 2 are from different slides because I stained many of them. However, result is negative and no negatively stained metastasis is visible in the figure 11 indicating that there are no metastases in this sample. Anti-Pan Cytokeratin staining result was negative too but there is shown some brownish epitopes in the sample. Consequently, the sample 235 JIMT is negative for both antibodies.

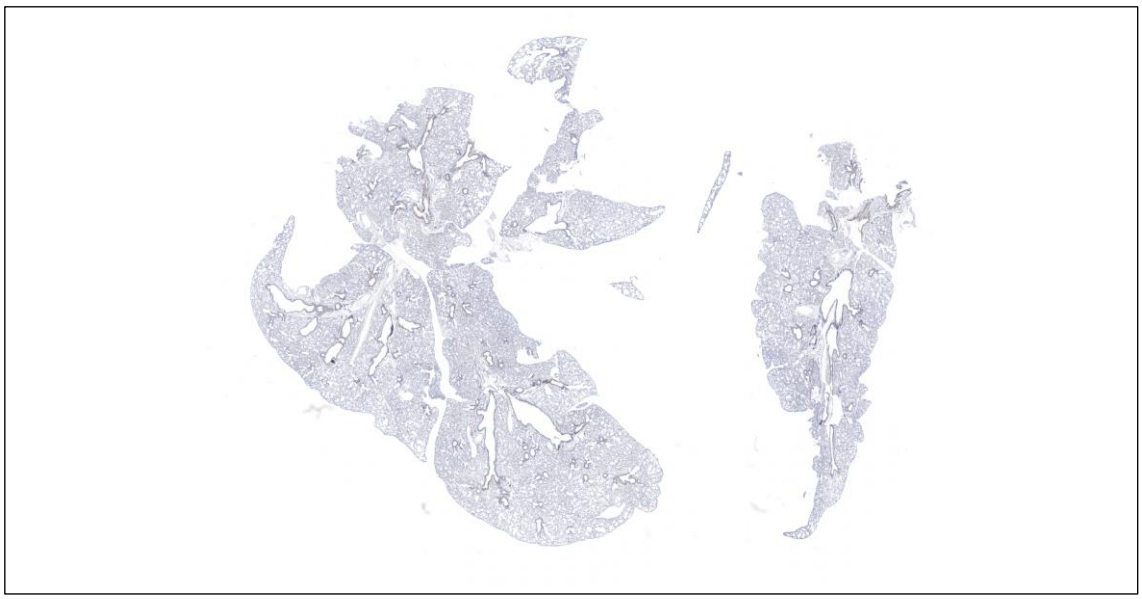

Figure 12. 235 JIMT. Anti-Pan Cytokeratin staining. Image 3 from Figure 11.

The figure 12 shows that in this staining had a cross reaction. It should be completely blue, but some lightly brown epitopes are visible, and no metastasis is shown. This is the same image, which is in figure 11, image 3. The cross-reaction occurs throughout the tissue sample. It is not in any specific spot nor place where the dye has concentrated clearly. I made the Anti-Pan Cytokeratin staining for this sample two times with the same concentration and incubation time and the result was the same at both times. It is possible that in this sample mice tissue had some similar antigens and antibody was binding to them.

235 JIMT sample had cross-reaction and it is possible that antibody was shown an affinity for identical or similar epitopes in mice lung tissue. Cross-reactivity was only detected in this sample and it was stained at the same time with other samples, indicating that it is not a staining error nor dependent on antibody concentration.

5.3.6 234 JIMT

Here is shown HER-2 staining result of sample 234 JIMT. Since I had a few technical problems the picture of Anti-Pan Cytokeratin staining is missing from this work. I have done it and know a result, so I told the result below.

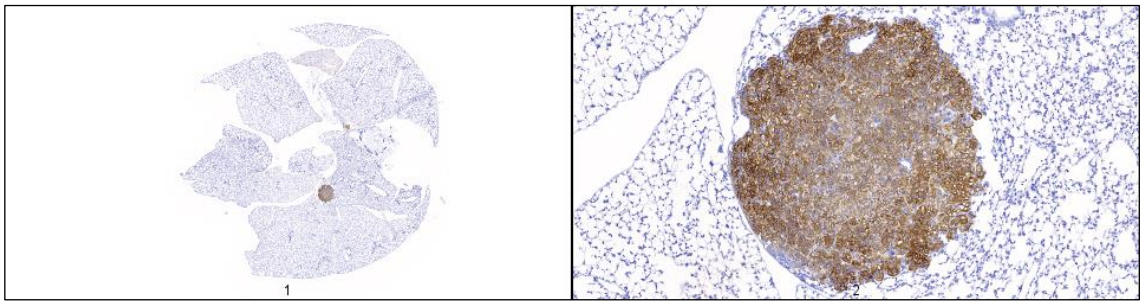

Figure 13. 234 JIMT. HER-2 staining. In images 1. is shown overview of the slide. In image 2 is shown positive stained metastasis.

As figure 13 is shown there were one big sized metastasis in the sample 234 JIMT. The result is strongly positive, and the staining is smooth. The metastasis in image 2 is circular, with a precise boundary and a dense structure. On the other side of the metastasis is a portion that is stained negative but belongs to the same tumor. Cell structure in this metastasis is dense and there are variations in cell sizes and shapes. Samples 234 JIMT Anti-Pan cytokeratin staining result is negative, but the metastasis is clearly distinguished

in the image. Unfortunately, because of some technical problems that image is missing, but it is available from Biomedicum.

6 Discussion

6.1 Results review and conclusions

HER-2 and cytokeratins are biomarkers that have been used in cancer and tumor diagnostics. HER-2 biomarker has been commonly used to help with the prognosis and assessment of cancer treatments to avoid mortality. HER-2 positive cancer is a more aggressive disease, with a higher relapse rate and increased mortality. (Gutierrez — Schiff 2011.) Consequently, we detected HER-2-positive cells in the mouse lungs, and those are from the human JIMT-1 breast cancer cell line that metastasized the mouse lung. In this work, there were three samples which were positive to HER-2. HER-2 positive stained metastases support the finding that HER-2 positive breast cancer is metastatic, and it inevitably affects the choice of cancer drugs.

HER-2 positivity indicates that cancer has a poor prognosis and is usually metastatic (Gutierrez — Schiff 2011). It shows in these staining, and the expression of metastases in the samples also indicates that HER-2-positive cancer is prone to metastasis. Metastases with HER-2 positivity are clearly from the JIMT-1 human cancer cell line. Since the HER-2 antibody was specific for human HER-2 expression, it is then clear that it is of human origin. Cytokeratin positivity supports poor prognosis but can also help in cancer treatment planning and drug selection as well as HER-2 (Karantza 2011). If the specific cytokeratin is known, it could help with localizing the primary tumor and assessing the response to drug therapy.

Anti-Pan Cytokeratin staining did not give as strong positive results as HER-2 staining, but as the controls showed, their positive control stained in the same manner as the positive samples. Because cytokeratins appear more accurately at the edges of metastases and, the example in smaller tumors, it may be possible that the cells are at different stages and express cytokeratins more strongly. All the sample slides are stained well when I compared them to the controls. During this work, a few staining failed, and it was likely that the failed slide dried too much during the antibody incubation phase, as the dye had accumulated as clumps on the slides. However, I repeated all the failed staining and did not use the failed images in my results.

The results of different samples indicate differences in metastases and the expression of HER-2 and Cytokeratin antigens used. All positively stained metastases shown in the figures have shown that the cell distribution of metastases is typical of cancer. Cancer cells are more tightly organized, and there are variations in cell sizes. The structure of the lung tissue is looser and fibrillary reticulated than the structure of the metastases, and it is visible in all images containing tumors. It is clear that metastases expressing the HER-2 antigen are from the JIMT-1 human breast cancer cell line. HER-2 antibody is specific for human epidermal growth factor 2, and the positive result of HER-2 staining is telling that it is human-based. Also, the Anti-Pan Cytokeratin antibody was specific to human cytokeratins, so all positive stained samples are from the JIMT-1 human breast cancer cell line. There was some cross-reactivity with anti-Pan cytokeratin staining, but the cross-reactions were distinguishable and could not have been confused with positively stained metastatic tumors.

6.2 Recommendations and further research

Breast cancers are classified into basic subtypes such as Luminal A, Luminal B, HER-2+ and basal-like and there are also mixed types of them. (Bray. et. al. 2018; Gutierrez — Schiff. 2011; Martin-Castillo et. al. 2015.) In cancer, cytokeratins are used as tumor markers in diagnostics because malignant tumors partially maintain specific keratin patterns associated with the original cells. Cytokeratins can be used to determine, for example, the prognosis of breast cancer and the tumor aggressiveness of cancer. (Gutierrez — Schiff. 2011; Karantza. 2011.) The basal HER2 + phenotype tumor, which expresses basal CK5 and CK6 in immunohistochemical staining, has been studied to be associated with aggressive disease, poor overall prognosis and predicted worsened disease-free survival. (Martin-Castillo et. al. 2015.) In the study Martin-Castillo et. al. (2015) concludes also that aggressive basal 2-HER2 + tumors that are positive for CK5 and CK6 are unlikely to respond to trastuzumab-based adjuvant or neoadjuvant therapy. (Gutierrez — Schiff. 2011) In the study by Chung et al. (2016) have also shown that Basal-HER2 + tumors are more likely to be resistant to trastuzumab and are associated with poorer survival. In the study, they are suggested that CK5 and CK6 expression is correlated with EGFR expression. Therefore, Martin-Castillo et. al. (2015) study and Chung et. al. (2016) study support each other. In this work, anti-Pan cytokeratin staining includes CK1, CK3, CK4, CK5, CK6, CK8, CK10, CK14, CK15, CK16, and CK19 and samples 218 JIMT, 236 2 / B JIMT, and M1 JIMT were positive for all or some of the above cytokeratins. As a result, it is not possible at this stage to distinguish which specific

cytokeratins are positive in these tissue samples, and if specific cytokeratins are to be determined, it will require other methods and thus further studies.

Cytokeratins are associated with deformability and invasive abilities, indicating that cytokeratins may influence cell shape and migration through interactions with the extracellular environment. Expression of cytokeratins CK8/CK18 may increases invasion, cellular adhesion and metastasis of breast cancer cells. In the study Karantza (2011) has presented that elevated CK8 levels may actively reduce the response to cancer treatment and together CK8 with breast cancer resistant protein may increase drug resistance, through different mechanisms. CK8 expression in breast cancer may also determine the aggressiveness of the tumor and it may decrease response to the cancer treatments. Gusterson et. al (2005) state in their study that CK17 regulates cell size and growth and is used to distinguish myoepithelial cells from luminal epithelium cells and CK17 expression in breast tumors is associated with poor prognosis and high tumor grade. CK5, CK14 and CK17 expression in breast cancer cells is also shown to be associated with poor prognosis. The data obtained from the studies are fascinating and can be utilized if specific cytokeratins are determined from samples.

6.3 Exploitation of the results

The use of animal models to understand human diseases following ethical principles is important. In the researches of diseases and their drugs, it is essential to be able to model the efficacy of the drug and, for example, cellular structures in animal models. (Lei — Ren — Wang — Liang — Tang 2016.) The JIMT-1 used is a human breast cancer cell line and was injected into mice hoping it would form primary tumors that metastasized to the lungs and this thesis proves it happened. HER-2 and cytokeratins may serve as prognostic markers and participate in the assessment of epithelial tumorigenesis as well as in the assessment of response to treatment (Karantza 2011; Martin-Castillo et. al. 2015). This characterization of the lung metastasis tumors in the lungs of mice will help the research group later use this model for studying the effect of different drugs in this model. However, the information on the progress of the study is confidential and therefore I cannot open it further in this context.

6.4 Reliability

The whole study has followed honesty, general diligence, and accuracy in the research work and the recording and presentation of results and the evaluation of studies and their results. Regarding laboratory work, especially staining, it is important to follow proper work procedures and methods during the study. During the implementation, I worked carefully and followed all the instructions of the methods I used. I received IHC staining instructions from the research group laboratory and I used Abcam general instructions for IHC staining. (Abcam: IHC staining protocol.) During the study, we optimize the Anti-Pan Cytokeratin staining, which increases the reliability of the results. HER-2 staining has been optimized in the past by others. I read the safety instructions for hazardous substances used in the work before working. I always wore a lab coat while working and nitrile gloves to protect against skin contact when processing hazardous substance. I worked in a laminar cabinet while working with hazardous substances such as xylene for example. All the hazardous substance waste was disposed of following the hazardous substance safety instructions and Biomedicum protocols.

While working in the laboratory, I wrote everything I did in the lab book and always read the instructions before working with the new device. Tissue samples must be properly processed before paraffin embedding. Working with mice and collecting and preparing the tissue samples was performed by other members of the research group. I did the slicing of the tissue sample blocks by myself and if I had any difficulty with the slicing, I asked help from Marja Ben-Ami, who has a long experience and expertise in tissue sections. When performing experimental staining, control samples must be included in all experiments for reliability, and thus the correctness of the staining can be ensured. I optimize one of the two IHC antigen stains and present control samples to demonstrate reliability in chapter 5.2, based on figure 2 and figure 3. The images in this file are promoted and cropped from the original images. All the images in this thesis are available in their original size and format.

6.5 Research ethics

All the references which I have used in this bachelor’s thesis are from trusted sites. I mainly chose fresh sources up to 10 years old, but some sources are older. The older sources which I have used are most discussions or basic knowledge that has not

changed during the time. Most of the sources are from the National Center for Biotechnology Information (NCBI) or Science Direct sites. I used Abcam sites for a source with the methods because we were using their products and they got a good IHC protocol manual. All the articles which I used for references were free to read. This produce text has not been plagiarized, I have checked my thesis with Turnitin-program, and I have referred to the used references properly.

This work has not included any human samples or any patient information. All the used specimens are from mice. Human skin control slides are from human skin tissue sample block and HUSLAB's pathology laboratory has given it without any patient information nor even knowledge of gender. The pathology laboratory has diagnosed the skin tissue to be healthy before giving it to the research group's laboratory. The results presented in this bachelor's thesis are shown as they are, without changing them to suit better the needs. We made a contract between me, the research group, and Metropolia University of Applied Sciences and it is signed at the beginning of this process. The research group has applied for and obtained all necessary permits before starting the study. I did not work with test mice during implementation, I did not even see an animal lab in Biomedicum, but there are a lot of ethical questions involved in the research use of mice.

Mice are used in medical researches because they have physiologically and phylogenetically similarities with humans for example the immune systems are similar. Mice researches have given much information in the adaptive immune system, T-cell receptor and understanding of different diseases and medicines during in the past century. (Perlman. 2016.) Mice are living creatures, so ethical content is important in mice researches and mice should be used in researches only within an ethical frame and under the laws. (Festing – Wilkinson 2007.)

Experiments on mice are scientific and necessary to achieve the research results. In this *in vivo* study mice are immunocompromised to achieve the best results. The research group has the needed project license from the national board of animal experiments. Always before starting new research that contains a test animal study, it must be obtained. The research will continue further, and its future is related to cancer medicines and treatments. This project follows the right ethical principles, responsibility and scientific practice and takes all these into account throughout the research. Scientific practice and experimental design help to minimize the use of test mice. (European parliament and the council of the European Union. Directive 2010/63/EU; Government Decree on

the Protection of Animals Used for Scientific or Educational Purposes. 564/2013; Perlman 2016.)

In Biomedicum the researchers which are using mice in the study and will work with them must do the course which gives the right to perform animal-related measures, design projects and procedures, to care for animals and killing animals. The course gives the right to work according to Act 497/2013, Decree 564/2013 and Directive 2010/63/EU. In Finland, the use of animals for research purposes is strictly regulated by law. Law 564/2013 lays down precise requirements for the facilities and operation of a laboratory for the use of animals that support the ethical treatment of animals. (Government Decree on the Protection of Animals Used for Scientific or Educational Purposes 564/2013; European parliament and the council of the European Union. Directive 2010/63/EU; University of Helsinki. LAS education and training 2020.) The European Union's Directive applies to all its Member States and Directive 2010/63/EU establishes the principles of replacement, reduction, and refinement. In principle, it is specified that the Member States shall ensure that: 1. Whenever possible, a reliable method or test strategy which does not include experimental animals shall be used. 2. The project should use as few experimental animals as possible without compromising its objectives. 3. The breeding, accommodation, care, and measures of experimental animals shall be carried out in such a way as to avoid or minimize the pain, suffering, distress and lasting harm to the animals. (Hobson-West 2016.)

6.6 Professional growth

The bachelor's thesis process started in the spring of 2019. I met Mark Barok for the first time at Biomedicum, Helsinki, in the fall of 2019. During the meeting, I got an introduction to the research of the research group and received the topic for my thesis. After receiving the topic, I started to explore and get to know the subject better and started writing a plan and gathering a theoretical knowledge base. The thesis was implemented between October 2019 and February 2020.

The methods and professional foundations learned in the laboratory work and during this degree supported me in doing this work. Particularly useful was a basic knowledge of laboratory work and principles of immunohistochemical staining. Immunohistochemistry was the main method in this work and all the performed tasks in the laboratory were related to it. Staining is the basic method used by a Biomedical Laboratory Scientists.

The skills and knowledge learned during the clinical histology and molecular genetics research course also proved useful in the thesis implementation. There are many basic methods used by research groups and it is easy to implement them with the profession and knowledge of a biomedical laboratory scientist. Therefore, research groups also need the profession of Biomedical Laboratory Scientist because they have a basic knowledge of the clinical laboratory methods, their use and basic knowledge of natural sciences and technology. However, working in a research environment differs in many ways from a basic clinical laboratory and it has been comprehensive to see such an employment opportunity as well. I learned a lot about working day planning and scheduling, as well as what tasks a biomedical laboratory scientist can have when working in a research team.

7 Acknowledgements

I want to express my gratitude to Mark Barok, for giving the topic to my thesis and guiding and helping me throughout the process. I would also like to thank all the members of the research group, you received me well and provided help when I needed it. I received professional guidance and advice throughout the process. I want to thank Marja Ben-Ami for guiding me in the laboratory work and helping me whenever I needed. I'm delighted and I appreciate receiving this experience of research work. I developed a lot professionally during the process and I got responsibility during the work and that taught me a lot.

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ErbB2 datasheet

invitrogen

Catalog # MA5-16348

ErbB2 (HER-2) Monoclonal Antibody (SP3)

Product Details	
Size	500 µL
Species Reactivity	Human
Published Species	Human, Mouse
Host/Isotope	Rabbit / IgG
Class	Monoclonal
Type	Antibody
Clone	SP3
Conjugate	Unconjugated
Immunogen	Recombinant protein encoding extracellular domain of human c-erbB2
Form	Liquid
Purification	Protein A
Storage buffer	PBS with 1% BSA
Contains	0.1% sodium azide
Storage Conditions	-20° C, Avoid Freeze/Thaw Cycles
RRID	AB_2537867

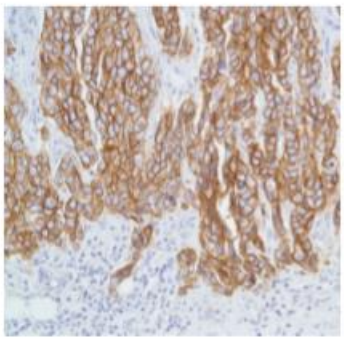
Applications	Tested	Dilution	Published
Immunohistochemistry (Paraffin) (IHC (P))	✓	1:100	10 Publications
Immunohistochemistry (IHC)	-	1:200	70 Publications
Immunocytochemistry (ICC)	-		3 Publications
Western Blot (WB)	-		2 Publications

Product Specific Information

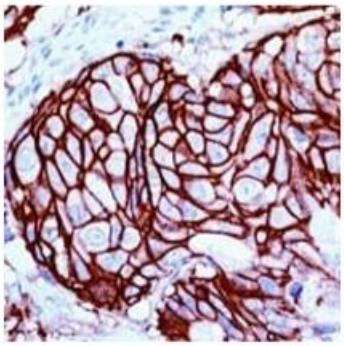
Heat-mediated antigen retrieval is recommended prior to staining, using a 10mM citrate buffer, pH 6.0, for 10 minutes followed by cooling at room temperature for 20 min. Following antigen retrieval, incubate samples with primary antibody for 30 min at room temperature. A suggested positive control is breast carcinoma.

Product Images For ErbB2 (HER-2) Monoclonal Antibody (SP3)

Product Images For ErbB2 (HER-2) Monoclonal Antibody (SP3)



ErbB2 (HER-2) Antibody (MA5-16348) in IHC
Immunohistochemical analysis of c-erbB-2/HER-2 using anti-c-erbB-2/HER-2 Monoclonal Antibody (Product # MA5-16348) in Breast Carcinoma Cancer Tissue. The recommened dilution for this antibody in immunohistochemistry applications is 1: 100.



ErbB2 (HER-2) Antibody (MA5-16348) in IHC
Immunohistochemical analysis of ErbB2 using a monoclonal antibody (Product # MA1-39544).

85 References

Immunohistochemistry (Paraffin) (10)

mAbs

Improved decision making for prioritizing tumor targeting antibodies in human xenografts: Utility of fluorescence imaging to verify tumor target expression, antibody binding and optimization of dosage and application schedule.

"MA516348 was used in immunohistochemistry - paraffin section to optimize in vivo and ex vivo fluorescence imaging for the preclinical development and characterization of therapeutic monoclonal antibodies"

Authors: Dobosz M,Haupt U,Scheuer W

Species
Human

Dilution
1:100

Year
2017

Oncology letters

Attempt towards a novel classification of triple-negative breast cancer using immunohistochemical markers.

"MA5-16348 was used in immunohistochemistry - paraffin section to classify triple-negative breast cancer into subtypes"

Authors: Liu YX,Wang KR,Xing H,Zhai XJ,Wang LP,Wang W

Species
Human

Dilution
1:200

Year
2016

View more IHC (P) references on thermofisher.com

Immunohistochemistry (70)

Breast cancer research and treatment

Increased CD4 and CD8-positive T cell infiltrate signifies good prognosis in a subset of triple-negative breast cancer.

"MA516348 was used in immunohistochemistry to explore the relationship between tumor-infiltrating lymphocytes and the clinical outcomes of triple-negative breast cancers"

Authors: Matsumoto H,Thike AA,Li H,Yeong J,Koo SL,Dent RA,Tan PH,Iqbal J

Species
Human

Dilution
1:200

Year
2016

Pathology, research and practice

The clinicopathological and prognostic significance of CD24, CD44, CD133, ALDH1 expressions in invasive ductal carcinoma of the breast: CD44/CD24 expression in breast cancer.

"MA5-16348 was used in immunohistochemistry to evaluate the expression of CD24, CD44, CD133 and ALDH1 in invasive ductal carcinoma of the breast"

Authors: Kapucuoglu N,Bozkurt KK,Bapnar ,Koçer M,Erolu HE,Akdeniz R,Akçil M

Species
Human

Dilution
Not Cited

Year
2015

View more IHC references on thermofisher.com

More applications with references on thermofisher.com

ICC (3) WB (2)

Anti-Pan Cytokeratin datasheet

abcam

Product datasheet

Anti-pan Cytokeratin antibody ab217916

1 Image

Overview

Product name	Anti-pan Cytokeratin antibody
Description	Rabbit polyclonal to pan Cytokeratin
Host species	Rabbit
Specificity	This antibody cocktail recognizes acidic (Type I or LMW) and basic (Type II or HMW) cytokeratins, which 67kDa (CK1); 64kDa (CK3); 59kDa (CK4); 58kDa (CK5); 56kDa (CK6); 52kDa (CK8); 56.5kDa (CK10); 50kDa (CK14); 50kDa (CK15); 48kDa (CK16); 40kDa (CK19).
Tested applications	**Suitable for:** IHC-P
Species reactivity	**Reacts with:** Human
Immunogen	**This product was produced with the following immunogens:** Recombinant full length protein within Human Cytokeratin aa 1-578. The exact sequence is proprietary. NCBI Accession No. 374454. Sequence: MSHQFSSQSAFSSMSRRVYSTSSSAGSGGGSPAVG SVCYARGRCGGGGYG IHGRGFGSRSLYNLGGSRSISINLMGRSTSGFCQGGGV GGFGGGRGFGVG STGAGGFGGGGFGGAGFGTSNFGLGGFGPYCPPGGI QEVTINQSLLEPLH LEVDPEIQRIKTQEREQIMVLNNKFASFIDKVRFLEQQN QVLQTKWELLQ QVNTSTGTNNLEPLLENYGDLRRQVDLLSAEQMRQNA EVRSMQDVVEDY KSKYEDEINKRTGSENDFVVLKKDVDAAYVSKVDLES RVDTLTGEVNFLK YLFLTELSQVQTHISDTNVILSMDNNRSLDLDSIIDAVRT QYELIAQRSK DEAEALYQTKYQELQITAGRHGDDLKNSKMEIAELNRT VQRLQAEISNVK KQIEQMQSLISDAEERGEQALQDAWQKLQDLEEALQQ SKEELARLLRDYQ AMLGVKLSLDVEIATYRQLLEGEESRMSGELQSHVSIS VQNSQVSVNGGA GGGGSYGSGGYGGGSGGGYGGGRSYRGGGARGRSG GGYGSGCGGGGGSYG GSGRSGRGSSRVQIIQTSTNTSHRRILE

Database link: Q7Z794

Recombinant full length protein corresponding to Human Cytokeratin aa 1-638. NCBI Accession No. 51350.
Sequence: MNRQVCKKSFSGRSQGFSGRSAVVSGSSRMSCVARSGGAGGGACGFRSGA
GSFGSRSLYNLGSNKSISISVAAGSSRAGGFGGGRSSCGFAGGYGGGFGG
SYGGGFGGGRGVGSGFGGAGGFGGAGGFGGPGVFGGPGSFGGPGGFGPGG
FPGGIQEVIVNQSLLQPLNVEIDPQIGQVKAQEREQIKTLNNKFASFIDK
VRFLEQQNKVLETKWELLQQQTTGSGPSSLEPCFESYISFLCKQLDSLLG
ERGNLEGELKSMQDLVEDFKKKYEDEINKRTAAENEFVGLKKDVDAAFMN
KVELQAKVDSLTDEVSFLRTLYEMELSQMQSHASDTSVVLSMDNNRCLDL
GSIIAEVRAQYEEIAQRSKSEAEALYQTKLGELQTTAGRHGDDLRNTKSE
IMELNRMIQRLRAEIENVKKQNANLQTAIAEAEQRGEMALKDANAKLQDL
QTALQKAKDDLARLLRDYQELMNVKLALDVEIATYRKLLEGEECRMSGEC
QSAVCISVVSNVTSTSGSSGSSRGVFGGVSGSGSGGYKGGSSSSSSSGYG
VSGGSGSGYGGVSSGSTGGRGSSGSYQSSSSGSRLGGAGSISVSHSGMGS
SSGSIQTSGGSGYKSGGGGSTSIRFSQTTSSSQHSSTK
Database link: Q01546

Run BLAST with [redacted] Run BLAST with [redacted] Run BLAST with [redacted] Run BLAST with [redacted]

Positive control IHC-P: Human skin tissue.

Properties

Form	Liquid
Storage instructions	Shipped at 4°C. Store at +4°C short term (1-2 weeks). Upon delivery aliquot. Store at -20°C long term. Avoid freeze / thaw cycle.
Storage buffer	Preservative: 0.05% Sodium azide Constituents: 0.05% BSA, 99% PBS
Purity	Protein A/G purified
Clonality	Polyclonal
Isotype	IgG
Light chain type	kappa

Applications

Our Abpromise guarantee covers the use of **ab217916** in the following tested applications.

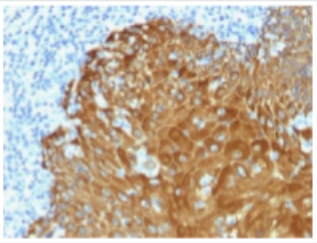
The application notes include recommended starting dilutions; optimal dilutions/concentrations should be determined by the end user.

Application	Abreviews	Notes
IHC-P		Use a concentration of 0.25 - 0.5 µg/ml. Perform heat mediated antigen retrieval with citrate buffer pH 6 before commencing with IHC staining protocol.

Target

Relevance	Cytokeratins, a group comprising at least 29 different proteins, are characteristic of epithelial and trichocytic cells. Cytokeratins 1, 4, 5, 6, and 8 are members of the type II neutral to basic subfamily. Monoclonal anti cytokeratins are specific markers of epithelial cell differentiation and have been widely used as tools in tumor identification and classification. Monoclonal Anti Pan Cytokeratin (mixture) is a broadly reactive reagent, which recognizes epitopes present in most human epithelial tissues. It facilitates typing of normal, metaplastic and neoplastic cells. Synergy between the various components results in staining amplification. This enables identification of cells, which would otherwise be stained only marginally. The mixture may aid in the discrimination of carcinomas and nonepithelial tumors such as sarcomas, lymphomas and neural tumors. It is also useful in detecting micrometastases in lymph nodes, bone marrow and other tissues and for determining the origin of poorly differentiated tumors. There are two types of cytokeratins the acidic type I cytokeratins and the basic or neutral type II cytokeratins. Cytokeratins are usually found in pairs comprising a type I cytokeratin and a type II cytokeratin. Usually the type II cytokeratins are 8kD larger than their type I counterparts.
Cellular localization	Cytoplasmic

Images



Immunohistochemistry (Formalin/PFA-fixed paraffin-embedded sections) - Anti-pan Cytokeratin antibody (ab217916)

Immunohistochemical analysis of formalin-fixed paraffin-embedded human skin tissue, labeling Cytokeratin using ab217916 at 0.5 µg/mL.

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