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DETECTION OF ENDOSIALIN IN HUMAN TUMORS BY IMMUNOHISTOCHEMISTRY

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ABSTRACT

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Endosialin, also named as tumor endothelial marker 1 (TEM1) or CD248, is a poorly known cancer associated glycoprotein which has been found to express in different kinds of tumors, but not in normal tissues. There are several suggested functions for endosialin, but the overall function, activating mechanisms, signaling pathways and regulation are still mainly unknown. In this study the expression patterns of endosialin in human tumors was analyzed by immunohistochemistry. The aims of the study were to find out the suitability of two endosialin monoclonal antibodies for immunostaining and to compare the expression of two endosialin antibodies between human tumor specimens and normal tissues.

The expression of endosialin was investigated by immunohistochemistry. Tumor specimens were processed and immunostained with two different endosialin monoclonal antibodies II-50/3 and VIII-16. The used immunostaining technique was indirect method. Secondary antibody was labeled with horseradish peroxidase. Staining intensity was investigated with light microscope and results were analyzed statistically.

In this study the results suggested that both the antibodies function well, but the overall staining intensity was bit stronger with antibody VIII-16. Moreover it was found that brain, colon and skin tissues stained more intensively than pancreatic tissues or hepatobiliary lesions. The strongest staining intensity was found in skin specimens. However endosialin functioned best in brain specimens where intense staining was observed in brain tumors and weak staining was observed in normal brain specimens. Correlation between staining intensity and different tumor grades was not found in this study.

According to the results of this study connection between endosialin and cancer was observed. This study indicates that endosialin could possibly in future serve as a tool in development towards better cancer treatment. Whether it has potential to become a specific molecular biomarker is uncertain, because in this study specificity and other typical features of molecular biomarkers were not found.

Key words: Tumor, immunohistochemistry, immunostaining, endosialin, TEM1, molecular biomarker.

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Endosialiini, TEM1 (tumor endothelial marker 1) tai CD248, on tällä hetkellä vielä melko huonosti tunnettu usein syöpään liitetty glykoproteiini. Endosialiinin on havaittu ilmentyvän voimakkaammin kasvainkudoksissa kuin normaaleissa kudoksissa. Sen toimintaa on yritetty selvittää lukuisissa tutkimuksissa, mutta esimerkiksi aktivaatiomekanismeja, solunsisäisiä signalointireittejä tai säätelyä ei tästä huolimatta tunneta vielä varmuudella. Tässä tutkimuksessa endosialiinin ilmentymistä tutkittiin erilaisissa ihmisen kasvaimissa immunohistokemiallisesti. Tutkimuksen tavoitteina oli selvittää kahden monoklonaalisen endosialiinivasta-aineen soveltuvuutta immunovärjäykseen ja vertailla vasta-aineiden ilmentymistä ihmisen kasvainkudosten ja normaalien kudosten välillä.

Endosialiinin ilmentymistä tutkittiin immunohistokemiallisesti. Kasvainkudosnäytteet käsiteltiin ja immunovärjättiin kahdella eri monoklonaalisella endosialiinivasta-aineella II-50/3 ja VIII-16. Käytetty immunovärjäystekniikka oli epäsuora menetelmä. Sekundaarivasta-aine oli leimattu piparjuuriperoksidaasilla. Immunovärjäyksen intensiteettiä tutkittiin valomikroskoopilla ja saatuja tuloksia tarkasteltiin tilastollisella analyysillä.

Saatujen tutkimustulosten perusteella kumpikin immunovärjäykseen käytetyistä monoklonaalisista endosialiinivasta-aineista toimi hyvin. Kaiken kaikkiaan värjäytyminen oli hieman voimakkaampaa vasta-aineella VIII-16. Lisäksi havaittiin, että aivo-, paksusuoli- ja ihokudos värjäytyivät voimakkaammin kuin haima- ja maksakudos. Voimakkain värjäytyminen oli nähtävissä ihokudoksessa. Endosialiini toimi kuitenkin parhaiten aivokudoksessa, jossa kasvainkudoksen värjäytyminen oli selvästi voimakkaampaa kuin normaalin kudoksen. Tässä tutkimuksessa värjäytymisen voimakkuuden ja kasvaimen pahanlaatuisuusasteen välillä ei löydetty yhteyttä.

Tutkimuksessa saadut tulokset viittaavat endosialiinin ja syövän yhteyteen. Endosialiinilla saattaa olla merkitystä syöpätutkimuksessa ja tulevaisuuden syöpälääkkeiden kehityksessä. On kuitenkin epävarmaa, onko endosialiinissa ainesta syövän molekulaariseksi biomarkkeriksi, koska biomarkkeireiden tyypillisiä piirteitä kuten spesifisyyttä ei tässä tutkimuksessa havaittu.

Avainsanat: Kasvain, immunohistokemia, immunovärjäys, endosialiini, TEM1, molekulaarinen biomarkkeri.

CONTENT

ABBREVIATIONS

AA	Anaplastic astrocytoma, grade 3
bp	Base pairs
cDNA	Complementary DNA
CD248	Endosialin
CML	Chronic myeloid leukemia
DA	Diffuse astrocytoma, grade 2
DAB	3,3’-diaminobenzidine tetrahydrochloride
ECM	Extracellular matrix
EGF	Endothelial growth factor
EPC	Endothelial precursor cell
GBM	Glioblastoma multiforme, grade 4
GIST	Gastrointestinal stromal tumor
GS	Gliosarcoma, grade 4
HEMA	Hematoxylin
HER2	Human epidermal growth factor receptor 2
HNPCC	Hereditary nonpolyposis colorectal cancer
HRP	Horseradish peroxidase
IFN-α	Interferon-α
KO	Knockout
mRNA	Messenger RNA
MSI	Microsatellite instable
MSS	Microsatellite stable
PAGE	Polyacrylamide gel electrophoresis
PSA	Prostate-specific antigen
RT-PCR	Reverse transcriptase polymerase chain reaction
SAGE	Serial analysis of gene expression
SEM	Standard error of mean
TBS-TWEEN	Tris buffered saline with tween
TEM1	Tumor endothelial marker 1, endosialin
VEGF	Vascular endothelial growth factor
WT	Wild-type

1 INTRODUCTION

Histology is the study of the tissues (Greek words *histo*, tissue and *logos*, study) (Junqueira & Carneiro 2003, 1). It investigates microscopic structures and individual components of biological material (Stevens & Lowe 2000, 1). Tissues are comprised of several types of cells and extracellular matrix (ECM). Extracellular matrix consists of many kinds of molecules secreted by cells. The main functions of extracellular matrix are to support cells and transport nutrients to cells. (Junqueira & Carneiro 2003, 1.)

Histology has been used in diagnostic medicine for long. Histological examination of tissue samples is an important tool for histopathologic diagnostics of diseases. Through histological examination it is possible to distinguish normal tissue from diseased tissue and to determine which kind of disease is in question. For histological analysis tissues are usually processed and stained before microscopy, because they are usually too thick and also transparent for direct microscopy. Cells or specific tissue components are revealed in histological staining process. Histological staining provides examination of chemical nature of the tissues. (Alberts et al. 2008, 585.)

Immunohistochemical staining methods have considerably developed during the past few decades. Immunodiagnostic methods have had an important influence especially on development of histopathologic diagnostics. The use of immunological stains has become a routine diagnostic tool in most laboratories. (Carson 1997, 228.) Immunostaining exploits specific binding between antibody and antigen. Antibodies are proteins produced by immune system as a defense against infection. They are made of different forms with different binding sites, epitopes, that recognize target molecule, antigen, with high specificity. The location of antigen in sample can be precisely revealed with use of labeled antibodies. (Alberts et al. 2008, 588.)

Cancer is a genetic disease that arises as a consequence of genetic changes in DNA. According to American cancer society and international union against cancer there were 12 million cancers diagnosed last year with 7 million deaths worldwide. (Aggarwal et al. 2009, 1083-1084.) Despite the fact that knowledge of cancer has increased, the prevention and treatment is still deficient (Negm, Verma & Srivastava 2002, 288). Annual-

ly about 20 billion dollars are spent to find proper cures for cancer (Aggarwal et al. 2009, 1083.)

Initiation of cancer involves interaction of carcinogens with DNA. This interaction leads to somatic mutations and further to transformation of normal cells to tumor cells. The initiation is usually followed by promotion, the step in which cancer cells proliferate and form solid tumors. (Aggarwal et al. 2009, 1083.) There are two properties characteristic of cancer cells: Their reproduction differs from normal cell growth and division and they invade and colonize other cells' territories. Invasiveness is vital to cancer cells, because this ability allows cancer cells to enter blood or lymphatic vessels and form metastases. (Alberts et al. 2008, 1206.)

The earlier the cancer is diagnosed, the better treatment results will be achieved. At this moment cancer treatment has been concentrated on the early diagnosis of cancer and personalized medicine. (Negm et al. 2002, 288.) The early diagnosis of cancer has become possible through understanding of the molecular changes that happen in cells long time before the actual onset of cancer. These changes are called molecular biomarkers and they have been learnt to detect with novel improvements in technology. Detection of molecular biomarkers has helped us in development of new targeted anticancer drugs and therapies against different types of cancers. (Srinivas, Kramer & Srivastava 2001, 698.)

Endosialin, also named as tumor endothelial marker 1 (TEM1) or CD248, is a cancer associated protein. Endosialin has observed to express in tumor tissues, but not in normal tissues. The expression has been found in humans as well as in mice. Endosialin may have potential as a therapeutic target in developing cancer therapies. That is the reason why it attracts researcher's attention. (McFadyen et al. 2007, 363.)

The aim of the study is to identify the expression of endosialin in human tumors by immunohistochemistry. The study was carried out in summer of 2009 at the Institute of Medical Technology, University of Tampere, in the research group of tissue biology led by Professor Seppo Parkkila. This study was performed under the valued supervision of Professor Parkkila and laboratory technician Aulikki Lehmus.

2 REVIEW OF THE LITERATURE

2.1 Tumor diagnostics

A tumor is formed of abnormal mass of cells that grow and proliferate out of control. Tumor is said to be benign as long as its cells do not become invasive. Tumor is considered a cancer only when the tumor is malignant and its cells have gained the ability to invade surrounding tissues. (Alberts et al. 2008, 1206.)

Cancer is generally diagnosed when person get physical symptoms. After physical examination usually imaging studies are performed to produce pictures and to get more information of the tumor. Imaging studies such as x-rays, computed tomography scans, magnetic resonance imaging scans and positron emission tomography scans show for example the location and size of the tumor and whether it has spread in foreign tissues and formed metastases. Imaging studies have an important role in determining stage of the cancer. (National Cancer Institute 2004a.)

The purpose of cancer staging systems is to produce detailed information of cancer. Through cancer staging extent of the cancer spread and severity can be defined. There are lots of staging systems for many types of cancers. (National Cancer Institute 2004a.) Analyzed topics in cancer staging are often location of the primary tumor, size of the tumor, number of tumors, whether the cancer has spread to lymph nodes, cell type and presence or absence of metastases. (Cancer Treatment Watch 2005.)

According to normal cancer diagnostic pattern laboratory tests are subsequently taken to gain more information about the cancer. Laboratory tests may involve sampling of blood, urine or other fluids, but also tissue samples, or in some cases the entire tumor, are often removed from the cancer in a procedure called a biopsy. (National Cancer Institute 2004a; Cancer Treatment Watch 2005.) These tissue specimens are usually examined under a microscope by pathologists whose reports usually contain once again even more precise information about the cancer. Pathologists also determine the grade of the cancer. (Cancer Treatment Watch 2005.)

The tumor grade is a description of the tumor based on how abnormal the cancer cells look like under a microscope and how quickly the tumor is likely to grow and spread.

These grading systems are different for each type of cancer. Pathologists commonly use a four-degree system. The cells of grade 1 resemble normal cells and the grade 1 tumors are considered to behave in the least aggressive ways. The cells of grade 3 and 4 do not look like normal cells and tend to grow rapidly and spread fast. (National Cancer Institute 2004b.)

An individual cancer treatment plan for patient is developed and a prognosis is predicted according to the stage and the grade of the tumor. It is important to know the stage and the grade of the tumor to doctors to identify the best possible treatment for each case. Generally it can be said that the lower the tumor grade is the better the prognosis is. (National Cancer Institute 2004b.)

2.2 Molecular biomarkers

The most important thing in succeeded cancer therapy is the early detection of cancer. If the tumor can be identified at the earliest stage when it has not spread yet, the treatment will be easier and better response to treatment will be achieved. Typically tumors are detected not until patient gets symptoms. This fact signifies that we have still limited abilities in detecting cancers in their earliest stages and thus a lot to learn about it. (Negm et al. 2002, 288.)

It has been discovered that there are several molecular changes occurring in developing tumors a long time before first actual symptoms are detected. There are different changes in different types of cancers. It has been shown that these alterations take place in gene sequences, gene expression levels and protein structure or function. (Sidransky 2002, 210.) These alterations can be divided into genetic and epigenetic changes. Genetic alterations take place in DNA. These alterations include deletions, inversions, amplification and chromosomal translocations. Epigenetic changes are independent of DNA sequence. Epigenetic alterations include methylation, chromatin remodelling, histone phosphorylation and acetylation. (Aggarwal et al. 2009, 1084.)

These molecular alterations are considered as diagnostic markers or molecular biomarkers. In other words biomarkers are cellular indicators of the physiological state of a cell at a specific time. Active genes, protein products and other organic chemicals that cells make comprise the molecular signature of a cell. (Srinivas et al. 2001, 698; Sidransky

2002, 210.) An ideal biomarker should be easy to detect, measurable across the population and have an early alteration with high specificity. Biomarkers can also help in determining the sensitivity or resistance of cancer cells to therapies and identification of high-risk individuals before the onset of cancer (Srinivas et al. 2001, 698; Aggarwal et al. 2009, 1086.)

With help of molecular biomarkers it would be easier to detect the cancer at the earliest stage, make the diagnosis, understand the characteristics of the cancer, determine prognosis and monitor the progression of the disease and responses to selected treatments in future. By early detection and better understanding of the features of the cancer with help of molecular biomarkers it would be easier to individualize or personalize cancer treatments according to the type of the cancer or for patient's needs. Through this way better prognosis and survival would be possible (figure 1). (Sidransky 2002, 210; Jimeno & Hidalgo 2006, 787.)

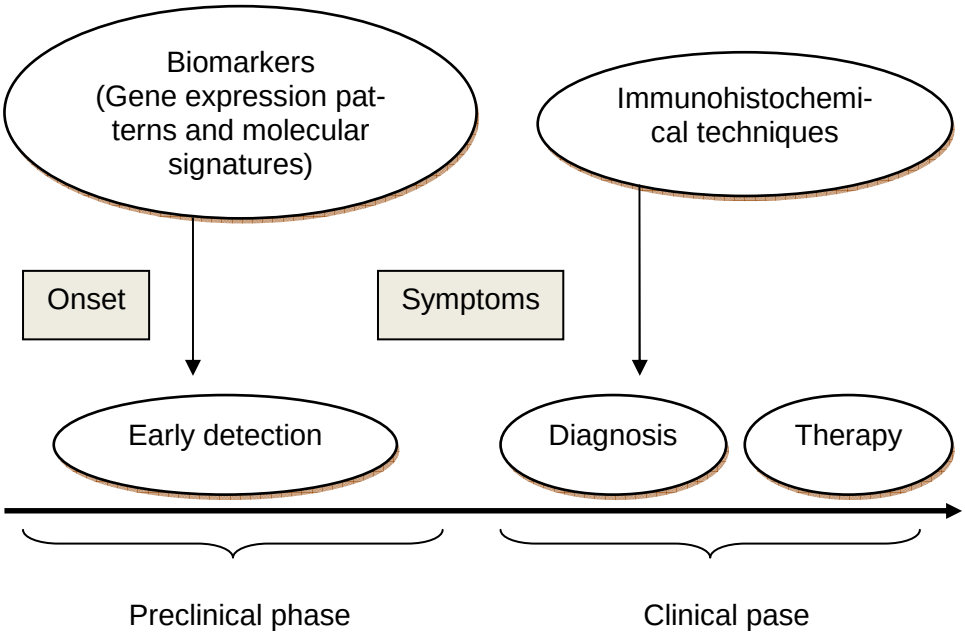


FIGURE 1. Detection and epidemiological progression of cancer. (Modified from Srinivas et al. 2001, 702)

2.2.1 Detection of molecular biomarkers

Before detection of molecular biomarkers the used biological specimen must be selected. These biological specimens can be tissue biopsies, body fluids, serum or plasma. Secondly the target for a molecular biomarker must be defined. Targets can be DNA, RNA or proteins, because all of them can be specific molecular biomarkers. (Jimeno & Hidalgo 2006, 787.)

Development of novel high-throughput technologies has increased our abilities to better understand and investigate the molecular mechanisms of cancers (figure 2) (Negm et al. 2002, 290). Advances in genomics and proteomics have helped us to detect these molecular changes using biomarkers. Genomics is a measurement of cell's gene expression profiles while proteomics detects the functioning units, proteins, of the expressed genes. (Srinivas et al. 2001, 700-701.)

Sample material for genomics is isolated DNA. Genomics exploits DNA microarrays, DNA, cDNA (complementary DNA) or oligonucleotide, to provide rapid information of gene-expression patterns. Serial Analysis of Gene Expression (SAGE) differs from microarrays in that the expression profiles identify expressed genes and quantify their expression levels. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) identifies individual transcripts synthesized from RNA. (Srinivas et al. 2001, 700.) Genomics provides a description of the transcriptional processes within cells (Negm et al. 2002, 290).

Sample material for proteomics is for example body fluids. Proteomics exploits two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), development of mass spectrometry techniques and information technology tools to detect potential cancer-associated proteins. (Srinivas et al. 2001, 701; Søreide et al. 2008, 44.)

Immunohistochemical method for detection of biomarkers is tissue microarray which enables the rapid evaluation of several biomarkers by immunohistochemistry. Tissue microarray is also suited to further examinations such as in situ hybridization and micro-dissection techniques. (Søreide et al. 2008, 44; Tissue Microarray Facility 2009.)

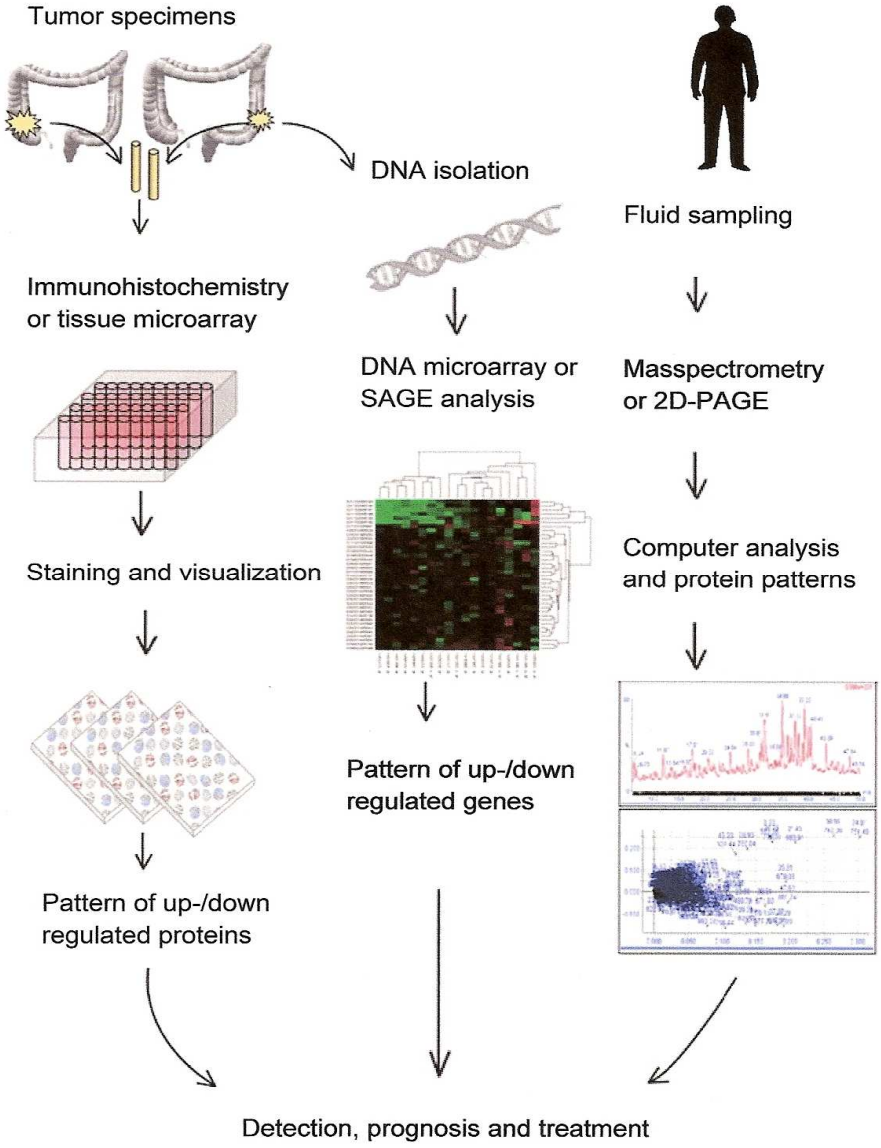


FIGURE 2. Diagnostic paths for diagnostics of molecular biomarker in cancer detection. (Modified from Søreide et al. 2009, 44)

2.2.2 Approved cancer biomarkers and targeted drugs

There have been difficulties in identification of biomarker for most cancers and at the present moment there are not decent biomarkers available for most of them. In addition, although biomarkers have been identified it does not ensure that they would function in desired way. (Aggarwal et al. 2009, 1086.) Two best known, approved and currently used biomarkers for detection of prostate cancers and breast cancers are prostate-specific antigen (PSA) and human epidermal growth factor receptor 2 (HER2). These

biomarkers are detected from urine or serum samples. For instance high serum levels of PSA are associated with prostate cancer, but cannot be used as a single indicator. (Negm et al. 2002, 290; Aggarwal et al. 2009, 1086.)

Several targeted anticancer drugs have been developed and approved for treatments of different kinds of cancers at present. A common feature in all of them is however uncertainty about patients' responses to these drugs. Trastuzumab (Herceptin) is a monoclonal antibody which is raised against an epitope in the intracellular domain of HER2. It is discovered that HER2 gene is present in 20-25% of breast cancers. The mechanism, how herceptin affects tumors is not fully understood. It has been suggested that herceptin might inhibit tumor growth by downregulation of receptors or by reducing the number of blood vessels of the tumor. Disadvantage of herceptin is the fact that patients often develop resistance to it after one year. Herceptin is well-tolerated drug and it is often used in combination with other chemotherapy drugs. (Aggarwal et al. 2009, 1086, 1088.)

Another targeted anticancer drug is avastin (Bevacizumab) which is a recombinant human monoclonal antibody raised against human vascular endothelial growth factor (VEGF) for metastatic colorectal cancers. It has been discovered that VEGF is expressed in half of the colorectal carcinomas. The effects of avastin in patients are unclear, but the disruption of tumor blood vessels is possible. Gleevac (Imatinib mesylate) is designed for treatment of chronic myeloid leukemia (CML) after normal treatment with interferon-α (IFN-α) have been failed. It has been reported to have only few minor side effects. (Aggarwal et al. 2009, 1090.)

According to Aggarwal et al. (2009, 1091-1092) individualized or personalized medicine for cancer treatment is important for two reasons: to increase efficiency and to decrease toxicity of drugs for patients. This is because some patients may be under-treated and some over-treated whereas some may not respond to treatments at all and some may suffer from toxic side-effects. The main idea of personalized treatment is to give the right drug at the right dose to the right patient. (Aggarwal et al. 2009, 1091-1092.)

2.3 Endosialin

Endosialin (alternatively named as TEM1 or CD248) was originally found in 1992 and described as the antigen of FB5 mouse monoclonal antibodies raised against cultured human fetal fibroblasts. At first it was considered as a specific marker of tumor blood vessels because of its expression in endothelial cells of malignant human cancers that were analyzed by immunohistochemistry. In analysis normal blood vessels and tissues lacked the FB5 expression. The protein was named as endosialin and the gene was located to chromosome 11q13. (Rettig et al. 1992, 10832.)

In 2000 gene expression patterns of human endothelial cells derived from blood vessels of normal and cancerous colorectal tissues were compared. Expression patterns were analyzed with SAGE-method. Many gene transcripts were found to be overexpressed in tumor endothelium. These genes were named as tumor endothelial markers (TEMs). Endosialin was independently discovered when *Tem1* gene was found to encode endosialin. (St. Croix et al. 2000, 1198, 1199.)

At present endosialin is described as a type I cell surface glycoprotein having a single-pass transmembrane α-helix. Endosialin consists of a 90 kDa core protein, but the molecule is highly modified by O-linked glycosylation comprising the mature form of 165 kDa. (Christian et al. 2001, 7408.) It has also been found that endosialin is expressed by tumor stromal fibroblasts and tumor blood vessel-associated perivascular cells (pericytes) rather than endothelial cells by themselves like earlier has been suggested (MacFadyen et al. 2005, 2569.)

2.3.1 Molecular structure of endosialin

Information of the molecular structure of endosialin was gained when partial amino acid sequence of endosialin was unraveled and its full-length cDNA was cloned. It became clear that endosialin molecule comprises of 2274 base pairs with an open reading frame and that the endosialin cDNA encodes for 757 amino acids core protein. According to bioinformatic evaluation endosialin can be classified as a C-type lectin-like protein. (Christian et al. 2001, 7408.)

Endosialin is composed of several functional domains (figure 3). A more N-terminal globular portion of the molecule consists of amino acids 30-360 and includes a N-terminal signal leader peptide, a C-type lectin domain, a Sushi domain and three endothelial growth factor (EGF) domains. A more C-terminal region consists of amino acids 361-757 and includes a mucin like region, a transmembrane region and a short cytoplasmic tail. N-terminal segment of endosialin was found to share 39% sequence identity to human thrombomodulin (CD141) and 33% sequence identity with human complement receptor C1qRp (Christian et al. 2001, 7410-7411.)

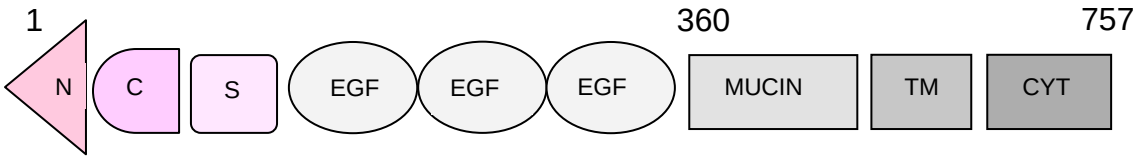


FIGURE 3. Domain architecture of endosialin molecule. The N-terminal signal leader peptide (N), the C-type lectin domain (C), The Sushi domain (S) and three EGF repeats (EGF) followed by the mucin-like region (MUCIN), the transmembrane section (TM) and the short cytoplasmic tail (CYT). (Modified from Christian et al. 2001, 7412)

2.3.2 Expression of endosialin

Despite of the fact that endosialin is expressed in tumors the expression has been detected also in normal endothelium. Normal expression of endosialin has been examined in developing mouse embryos, newborns and adults by immunohistochemistry and in messenger RNA (mRNA) levels by RT-PCR. Strong expression of endosialin was detected in developing mice embryos. In the embryos endosialin was expressed by fibroblasts and pericytes of developing vasculature. However there was a significant loss of expression in adults. Endosialin is thus downregulated during development of mouse leading to a conclusion that in normal adult tissues endosialin is not expressed. Expression studies of endosialin by RT-PCR revealed that expression levels of endosialin mRNA increase during the whole embryonic stage and rapidly decrease in newborns and adults. (MacFadyen et al. 2007, 363, 365, 367; Rupp et al. 2006, 1,4.)

In different studies the expression of endosialin was detected in various types of human tumors *in vivo* and *in vitro*. In a study of human brain tumors by immunohistochemistry

the expression of endosialin was localized to pericytes of blood vessels and stromal fibroblast cells. Normal tissue was negative for endosialin. The immunostaining reaction was detected in both primary and metastatic tumors. Brady et al. 2004, 1274, 1283.) Expression of endosialin in human breast tumor specimens by immunohistochemistry was studied by generating a panel of monoclonal antibodies against extracellular domain epitopes of human fibroblast cell surface proteins. The expression of endosialin was detected on tumor stromal fibroblast cells in every examined section. (MacFadyen et al. 2005, 2573.) Similar results were reported with human ovarian tumors, human colon carcinoma biopsies, cultured human neuroblastoma cells and mesenchymal stem cells. (Christian et al. 2008, 487, 493; Bagley et al. 2008, 187.)

An extensive and valuable study was made when expression of endosialin in *Tem1* knockout (KO) and wild type (WT) mice was investigated. Knockout is a genetic technique by which function of a selected gene is completely inactivated or deleted (Alberts et al. 2008, 566). Wild-type is normal nonmutant form of organism. The study firstly showed that *Tem1* KO mice developed normally and were completely healthy having a normal vasculature and wound healing capability. (Nanda et al. 2006, 3351.)

Human colorectal cancer cells were transplanted to abdominal sites of KO and WT mice. Interestingly there was a reduction in tumor growth, invasiveness and metastasis in tumors transplanted to abdominal sites of KO mice compared to WT mice. It was demonstrated that *Tem1* gene is not a requirement for tumor angiogenesis, but optimal growth, invasion and metastasis of tumors depend on the expression of endosialin. Statistically survival of KO mice of tumor growth period was 89% compared to 11% for WT mice. In addition tumors in KO mice grew more slowly with fewer metastases and smaller tumor volume. (Nanda et al. 2006, 3351, 3352.)

2.3.3 Functions of endosialin

The function of endosialin is not known for certain, but there are few reasonable suggested functions. The main function of endosialin is still under high investigation, because for example molecular functions and intracellular signaling pathways are still unknown. A full understanding of functions of endosialin in normal development and cancer is not possible without understanding first its molecular mechanisms and func-

tions that control its expression. (Rupp et al. 2006, 10; Ohradanova et al. 2008, 1348, 1353.)

The expression of endosialin is localized to the cell surface. For this reason it is expected that the function of endosialin is mediated through direct cell to cell interactions rather than through paracrine system. (Nanda 2006, 3354.) Endosialin is restricted to fibroblasts where it presumably functions as a cell surface receptor to bind ligands found in extracellular matrix (ECM) (McFadyen et al. 2005, 2574).

One suggested function of endosialin is the interaction with ECM proteins. In other words it is suggested that endosialin can adhere to the supporting proteins of ECM such as collagen and fibronectin and thus mediate the cell adhesion and migration through ECM. Interactions between endosialin and fibronectin, and collagen I and collagen IV have been found. These ECM proteins serve as ligands for endosialin. (Tomkowicz et al. 2007, 17969.)

2.3.4 Endosialin as a molecular biomarker

Expression of endosialin is often linked to angiogenesis. Firstly because endosialin is expressed in pericytes that are closely connected to endothelial cells during formation of blood vessels and secondly because of the expression of endosialin found in vasculature of developing mice embryos. (MacFadyen et al. 2007, 363; Bagley et al. 2008, 180.) Angiogenesis occurs during normal physiological development and under pathologic conditions like tumor growth (Bagley et al. 2008, 2536). Mostly it is a consequence of hypoxia in developing tissue. It has been found that hypoxia induces the expression of endosialin. This induction occurs through a mechanism that involves hypoxia-inducible factor-2 (HIF2) which has been earlier independently linked to regulate a part of genes involved in angiogenic responses. (Ohradanova et al. 2008, 1348, 1353.)

It has been suggested that blood vessel development may involve endothelial precursor cells (EPC). On the other hand it means that angiogenic process in cancer can involve EPC that express endosialin. It has been found that endothelial genes are up-regulated in both tumor vasculature and in EPC cells. Through investigations it has been observed that the expression of endosialin increased in EPC cells compared to mature endothelial

cells. This indicates that endosialin is connected to the early stages of angiogenesis. (Bagley et al. 2008, 2536.)

It has been shown that endosialin plays an important functional role not only in angiogenesis but also in tumor progression and in tumor stroma formation, because KO mice dramatically changed tumor growth patterns and reduced metastasis. Thus it has been suggested that interactions between endosialin and tumor cells may induce the production of other proteins in tumor cells or stroma that can help the tumor to grow and invade surrounding tissues. (Nanda et al. 2006, 3354.)

One specific ligand for endosialin has been identified. An extracellular ligand for specific endosialin interaction was named as Mac-2 BP/90K. It has been demonstrated that interaction of endosialin and Mac-2 BP/90K ligand mediates and regulates the adhesion and migration of tumor cells that express endosialin in ECM of tumor. Through experiments it has been shown that expression of Mac-2 BP/90K ligand correlates with distant metastases. Therefore Mac-2 BP/90K ligand seems to be functionally linked to tumor development and progression. (Becker et al. 2008, 3059, 3064-3065.)

2.3.5 Therapeutic prospects

Generation of anti-endosialin antibodies is widely ongoing. Antibodies, inhibitors and antibody-toxin conjugates are generated to, either directly or indirectly, influence the angiogenesis through different mechanisms. In addition single-chain antibody fragments directly towards the extracellular domain of endosialin are also produced. Antibody targeting therapies are evolving field in cancer treatment with a view to generate modern, effective and strictly targeted cancer therapies. (Bagley et al. 2008, 187; Bagley et al. 2008, 2544.)

Anti-endosialin antibodies may have potential for prevent migration of tumor cells and thus prevent the development of cancer. It could also be possible, with appropriate agent, to interfere with the growth of blood vessels during angiogenesis and prevent tumor development. Inhibition of pericyte function by targeting endosialin with a monoclonal antibody may provide a new strategy in which vascular destabilization and inhibition of angiogenesis may be possible. On the other hand the disruption of peri-

cyte-endothelial cell interactions could also serve a link to interfere the whole development of cancer. (Bagley et al. 2008, 187.)

In practice for example blocking the migration of EPC cells with anti-endosialin antibodies could be useful in preventing cancer growth and early angiogenesis (Bagley et al. 2008, 2536). On the other hand by targeting the endosialin-Mac-2 BP/90K ligand complex it might prevent the adhesion and migration of tumor cells and that way also prevent the metastatic spread of cancer (Becker et al. 2008, 3059).

2.4 Immunohistochemical methods

Immunohistochemistry is a routine tool in many laboratories. It combines anatomical, immunological and biochemical understanding and techniques to identify specific tissue components by specific binding between labeled antibodies and antigens. Immunohistochemistry provides for visualization the distribution and localization of cellular components within tissue. (Thermo Scientific 2009.)

Immunohistochemistry is a traditional diagnostic tool in identification and detection of cancer. These techniques are important for prediction of tumor behavior and prognosis. (Srinivas et al. 2001, 698.) Basic immunohistochemical examination includes sample obtainment, tissue processing, production of thin tissue sections, staining and microscopic analysis (figure 4).

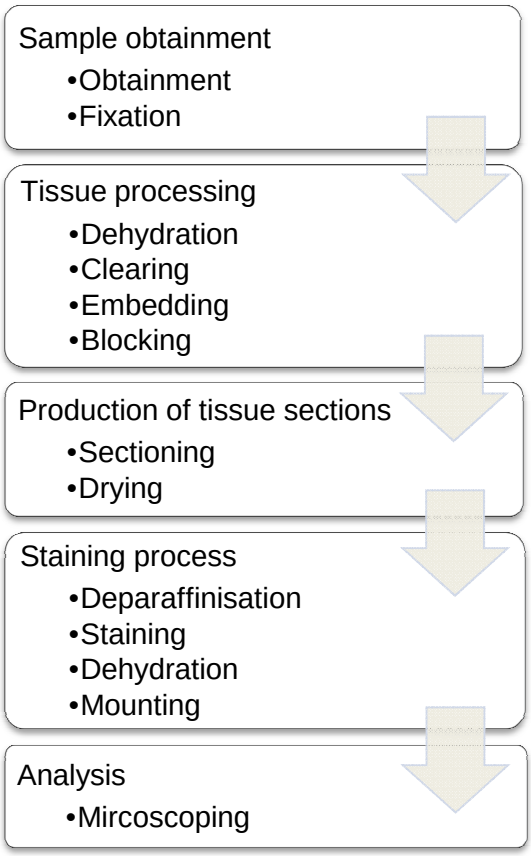


FIGURE 4. The steps of typical immunohistochemical examination.

2.4.1 Tissue sample obtainment

For diagnosing diseases it is remarkably important to obtain small tissue samples from human body. There are lots of simple and different techniques for the sample obtainment. Tissue sample can be obtained from several areas of the body. For instance samples from brain, eye, breast, liver, kidney, bone, bone marrow or muscle can be obtained by direct needle biopsy whereas samples from respiratory tract, alimentary tract or urinary tract can be obtained by endoscopic biopsy using endoscopic tubes. Scalpels are suitable for obtaining samples from directly accessible tissues such as skin, mouth, larynx or nose. (Stevens & Lowe 1997, 1.)

An alternative tissue obtainment technique is frozen sections. This technique is exploited especially during surgeries when information about the biopsy is needed as soon as possible. The technique allows a rapid preparation of sections without going through the regular tissue processing steps. The idea is to fix tissue sample by rapid freezing.

The use of isopentane and liquid nitrogen for rapid freezing has been found to produce best sections. After freezing tissue samples are ready for sectioning in cryostat, which is a special microtome, and staining with appropriate dyes. Frozen section technique can also be exploited when cellular lipids and enzymes are detected from tissues, because regular tissue processing would easily destroy them. (Carson 1997, 40-41, 52-52; Junqueira & Carneiro 2003, 2; Ross & Pawlina 2005, 4.)

2.4.2 Fixation

Most tissues are fixed before examination under microscope. Aims of the fixation are to prevent autolysis and bacterial attack, to prevent changes in shape or volume in tissues during any subsequent steps and to preserve tissues in a condition as close in their living state as possible. Fixation is performed by stabilizing proteins either by chemical (fixative solutions) of physical (heat, microwaves) means. Primary methods for fixation are the use of one or more chemical reagents. (Carson 1997, 2; Bancroft & Stevens 1999, 23.)

Fixative solutions are mostly classified as additive or nonadditive and coagulant or noncoagulant. Additive fixatives such as formaldehyde, glutaraldehyde, picric acid and osmium tetroxide change the tissue by chemically linking themselves to it. Nonadditive fixatives like acetone and alcohols do not add themselves to the tissue but function through precipitation or coagulation of proteins. Coagulant fixatives such as alcohol and picric acid create a network in tissues allowing solutions to easily penetrate into the interior of the tissue. Noncoagulant fixatives like formaldehyde, glutaraldehyde and osmium tetroxide create a gel making the penetration of solutions difficult. (Carson 1997, 2-3; Bancroft & Stevens 1999, 23-24.)

The most widely used fixative solution is formaldehyde. Commercially available solutions under trade name formalin contain 34-40% gas by weight. Formaldehyde is commonly used as neutral buffered formalin which contains 40% formaldehyde buffered to pH 7 with phosphate buffer. In aqueous solutions formaldehyde exists as its monohydrate, methylene glycol. Formaldehyde is additive and noncoagulant fixative and causes proteins to become insoluble by reacting with amino groups on the side chains of amino

acids. The reaction is reversible and pH-dependent. (Carson 1997, 8-10; Bancroft & Stevens 1999, 24-25, 35.)

2.4.3 Tissue processing

Tissue processing includes dehydration, clearing and embedding steps performed either manually or automated. Tissue processing is at present mainly automated. The automated process is similar to manual processing and contains same steps. First tissue processing step is called dehydration. It means removal of aqueous fixative fluids like water in tissues because embedding media will not infiltrate tissue containing any water. Dehydration is the most critical step of tissue processing because faults in this step are difficult to correct later (Bancroft & Stevens 1999, 53.) The most widely used dehydrating agent is ethanol. It is hydrophilic and miscible with water and with many organic solvents. It functions by attracting water from the tissues. Ethanol is delicate causing very little damage to tissues. Dehydration process is performed with series of increasing concentrations of ethanol starting from 70% and ending to absolute alcohol. (Carson 1997, 26-27; Bancroft & Stevens 1999, 49.)

Dehydration step is followed by clearing step. Clearing agents must be miscible with both the dehydrating agent (ethanol) and the embedding medium, frequently paraffin. The most widely used clearing agent is xylene. It removes the ethanol used in dehydration step, gives tissues a translucent appearance and makes tissues more receptive to the embedding medium. Too long clearing time hardens tissues leading to problems with tissue processing. (Carson 1997, 28; Bancroft & Stevens 1999, 50.)

Subsequently tissues are infiltrated with supporting embedding medium. The purpose of embedding is to hold proper relationship between cells and intracellular structures when sectioned. The most popular embedding medium is paraffin wax which is an inert mixture of hydrocarbons. The melting point has an effect on properties of paraffin wax. Usually waxes of melting point 54-58°C are used. (Carson 1997, 31-32; Bancroft & Stevens 1999, 51-52.)

Embedding is performed usually in a vacuum oven by immersing tissues to melted paraffin. Temperature of paraffin should be 2-4°C above its melting point. Vacuum helps

paraffin to infiltrate tissues. When immersing tissues to embedding medium paraffin penetrates tissues and removes residual air bubbles. Exposure to embedding medium is continued until air bubbles do not anymore come up. (Carson 1997, 32; Bancroft & Stevens 1999, 52-53.)

Tissue processing is followed by blocking which means enclosing tissue in paraffin wax. Blocking can be counted as a part in tissue processing. Blocking system comprises of melted paraffin wax dispenser, cold plate and heated area for molds and infusion of blocks. In blocking melted paraffin wax is dispensed into a preheated mould. Tissue specimen and tissue cassette are placed in mould. Mould is filled with melted paraffin wax, transferred to cold plate and allowed to cool down and solidify into block. Mould is removed from solidified block and block is ready for sectioning. (Bancroft & Stevens 1999, 52.)

There are few occasions when paraffin processing cannot be used in tissue processing and blocking: i. processing agents remove or damage investigated tissue components, ii. thinner sections are desired, iii. heat (with paraffin 56-60°C) would affect or damage tissue or iiii. paraffin do not offer as hard support as needed. Substitute processing and blocking agents for paraffin are for example other waxes like polyethylene glycol and resins like acrylic. For instance resins play an important role in electron microscopy when thinner sections and higher resolutions are needed. (Bancroft & Stevens 1999, 56-57.)

2.4.4 Production of tissue sections

So that the examination under microscope can take place thin tissue sections needs to be produced out of tissue blocks. Microtomes are instruments that allow production of sections with adjusted thickness; usually 5µm. Rotary microtomes are the most popular ones and the most suitable choice for paraffin embedded tissues. (Bancroft & Stevens 1999, 58.)

Requirements for thin tissue sections are proper temperature of the block, a sharp blade and suitable sectioning speed. Disposable blades provide a very sharp cutting edge and have replaced the old steel knives. The best sectioning speed depends on many qualities

such as softness of the tissue, air temperature, humidity, size of tissue and thickness of the sections. Unsatisfactory sections depend primarily on blunt blade or not cold enough temperature of block. (Bancroft & Stevens 1999, 60.)

2.4.5 Staining process

Completely dry tissue sections are deparaffinised before staining. Deparafinisation means removal of paraffin found in tissue sections. Any water left in tissue will lead to incomplete deparaffinisation and uneven staining. Deparaffinisation process is started with xylene, followed by series of decreasing consentrations of alcohols and ended up in distilled water. Xylene will remove paraffin while alcohols will remove xylene from sections. (Carson 1997, 57.)

Cells and their intracellular material are generally colorless. Staining is needed to visualize localization of cellular component within tissues in the light microscope. Staining can be made manually or automatically using some sort of staining automat. Stain uptake is due to dye-tissue or reagent-tissue affinities. Affinity describes the attractive forces which bind dye to tissue. On the other hand if tissue has a high affinity for a dye it usually becomes intensively stained. The dye is bound to tissue mostly by ionic, covalent or hydrogen bonds with help of van der Waals attractions. (Bancroft & Stevens 1999, 81; Histology and cell biology 2003.)

The dyes bind to certain tissue constituents selectively depending on the nature of the dye and the chemical composition of the tissue. There are positive and negative net charges found both in tissue components and dyes. If a dye ion has positive net charge it is cationic and also called a basic dye. If a dye ion has negative net charge it is anionic and also called an acid dye. If a tissue component has negative net charge, it will have affinity for basic dyes and vice versa. For example DNA and RNA will stain with basic dyes whereas cytoplasm and collagen stain with acid dyes. Usually more than one dye is used in staining process for increase of clearance. The first dye stains wanted tissue components selectively while the second dye is used as contrasting color to stain other tissue or cellular components. This is called counterstaining. (Carson 1997, 89; Histology and cell biology 2003.)

The following step for staining process is another dehydration process. The intention of dehydration is to remove water from tissue with series of increasing concentrations of alcohols ending up in xylene, which removes remaining alcohol. After dehydration cover glasses are applied to stained sections using some type of mounting medium. The step is called mounting. Cover glasses help to preserve stained sections and enable better microscopic analysis. (Carson 1997, 26, 107.)

2.4.6 Dyes

There are lots of different dyes. However all dyes are organic compounds and often also benzene derivatives. According to Carson (1997, 89) a group in a dye molecule conferring the color is called a chromophore. A common feature for every chromophore is that they are easily reduced. A chromogen is a benzene derivative containing a chromophoric group. A compound containing a group of chromophore or chromogen may not necessarily act as a dye and be able to combine with the substance to be colored. An ionizing group called auxochrome, most commonly amino group, is in some cases required to enable the dye to link to the tissue. (Carson 1997, 87, 89.)

An example of dyes is hematoxylin which is a natural color extracted from the heartwood of the tree *Haematoxylin campechianum* and used for nuclear staining. Hematoxylin is not itself a dye but the oxidation product hematein acts as a natural dye. Oxidation product can be produced from hematoxylin either with natural oxidation or chemical oxidation. Chemical agents used for oxidation are for example sodium iodate (Mayer's hematoxylin) or mercuric oxide (Harris' hematoxylin). (Carson 1997, 90-92.)

Hematein is anionic and has a poor affinity to tissue. That is why a presence of mordant is needed to nuclear staining. The most widely used mordants for hematoxylin are aluminium, iron and potassium salts. The mordant enables the binding of dye-mordant complex to anionic tissue sites as chromatin and nuclei. Hematoxylin is the most widely used in nuclear counterstaining, because it provides a good distinction between nuclei, cytoplasm and extracellular constituents. (Bancroft & Stevens 1999, 99, 101; Histology and cell biology 2003.)

2.4.7 Immunostaining techniques and enzyme labels

By immunohistochemical techniques it is possible to identify tissue constituents, antigens, by exploiting the specific interactions between antibodies and antigens. Immunohistochemical staining methods are based on the use of labeled antibodies. Labels are used for visualization of antigens found in tissues. Antibodies can be labeled either with enzymes, colloidal metals, fluorescent labels or radio labels. Enzymes are the most widely used labels in immunohistochemistry. Commonly used enzymes for labeling are horseradish peroxidase (HRP), alkaline phosphatase, glucose oxidase and beta-D-galactosidase. Incubation of enzyme-conjugated antibodies in presence of substrate and chromogen produces stable and colored reaction product suitable for light microscopic examination. (Bancroft & Stevens 1999, 438.)

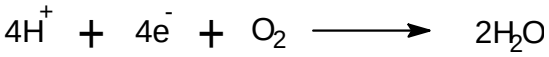
There are various immunostaining techniques used to detect presence of antigen in sample. However the most common techniques are direct and indirect methods. In direct technique label is conjugated to the primary antibody which detects antigens found in tissue. The advantage of direct technique is simplicity and the disadvantage low sensitiveness. Indirect method uses two antibodies, primary and secondary, to detect antigens (appendix 1, figure 1). Primary antibody is unlabeled and detects the antigens found in tissue. Labeled secondary antibody is followed against the primary antibody. Indirect technique is more sensitive technique compared to direct technique. (Bancroft & Stevens 1999, 439, 441-442.)

Immunohistochemical staining using enzyme-labeled antibodies results from the reaction between enzyme and its substrate. In the reaction soluble substrate with enzyme produces an insoluble and colored reaction end-product. Enzymes can be used in immunohistochemical staining instead of dyes. The intensity of formed colored reaction end-product should correlate to the concentration of the primary antibody and the tissue antigen. Enzymatic activity depends on concentration of enzyme and substrate, buffer, pH, temperature and light source. Variety of enzyme conjugated antibodies and chromogens are commercially available. (Thermo Scientific 2009.)

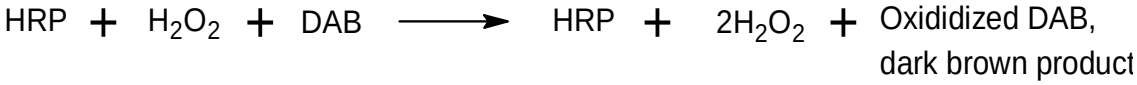
Large and significant group of enzymes used in immunostaining are oxidoreductases. This category includes oxidases, peroxidases, dehydrogenases and diaphorases. Peroxidases catalyse oxidation of its substrate by removing hydrogen. The hydrogen acceptor

for the hydrogen atoms is always molecular oxygen leading in the formation of water, chemical reaction 1A. In the oxidation reaction of HRP conjugated secondary antibody HRP is an enzyme while hydrogen peroxidase is a substrate and 3,3'-diaminobenzidine tetrahydrochloride (DAB) is a chromogen. Reaction produces oxidized DAB, insoluble dark brown colored reaction end-product visible for naked eyes and suitable for examination under light microscope, chemical reaction 1B. (Carson 1997, 254.)

A



B



CHEMICAL REACTION 1. Enzymatic reactions of peroxidases. A. Oxidation reaction of peroxidases. B. Enzymatic oxidation reaction of HRP involving substrate and chromogen. (Modified from Carson 1997, 254; Krysko et al. 2006, 2020)

In enzyme-labeled immunostaining it is important to block the endogeneous peroxidase activity and reactive sites found in tissues. If endogeneous peroxidase activity is not blocked, cellular components such as enzymes could result in unwanted signals and false positive staining result. Blocking of reactive sites found in tissues helps to avoid nonspecific binding of antibodies and also prevents false positive staining result. In HRP-technique the most widely used blocking agent for endogeneous peroxidase activity is hydrogen peroxidase and for reactive sites normal serum or colostrum. (Thermo Scientific 2009.)

3 AIMS OF THE STUDY

This study was focused on poorly known cancer associated protein, endosialin. The aim of the study was to investigate expression of endosialin in different human tumors and to identify the expression of endosialin by immunostaining. Specific aims of this research were

i. to characterize the function and suitability of novel endosialin specific antibodies for immunostaining,

ii. to investigate endosialin expression levels in tumor specimens.

4 MATERIALS AND METHODS

4.1 Sample material

Sample material for this study consisted of considerably large amount of tissue samples. Samples were selected from as many different tissues as possible in order to have a highly presentable sample material so that description of endosialin expression pattern would be possible in large scale. Tissue specimens from colon and pancreas were obtained from Jyväskylä central hospital. Tissue specimens from liver were obtained from Hospital district of Helsinki and Uusimaa. Tissue specimens from the brain, colon, skin, esophagus, antrum, duodenum, corpus and rectum were obtained from Tampere university hospital. Diagnosis with every specimen was known and the tumor grade of brain- and colon specimens was also defined.

All of the samples obtained from Jyväskylä central hospital and Hospital district of Helsinki and Uusimaa were formalin fixed and paraffin embedded, already sectioned and mounted on microscopic glass slides. The samples from Tampere university hospital were formalin fixed and paraffin embedded tissue blocks. For these tissue specimens sectioning and drying was performed before staining process.

4.2 Sectioning and drying

Before sectioning of tissue blocks both hot and cold water baths were filled with distilled water and the blocks were cooled down to proper temperature on a cold plate (+4°C). Sectioning was performed with microtome (Microm, GmbH, HM310, Walldorf, Germany) using disposable blades. Tissue block was placed on microtome so that the blade reached the whole surface of the block. Extra paraffin was trimmed away with thickness adjusted to 20µm. Before actual sectioning the thickness was adjusted to 5µm which was the desired thickness of sections.

Tissue sections were gained by rotating the microtome screw manually. The cut sections were floated onto the cold water bath containing room temperature water. By floating out sections the wrinkles were straightened. Sections were transferred to hot water bath

(+45°C) to achieve the expansion of sections to their original sizes. From hot water bath sections were transferred to microscopic glass slides and allowed to dry and rest over night. Drying of the sections was carried out in an oven (+60°C). Drying time was approximately two hours.

4.3 Immunostaining process

Deparaffinisation process was performed in staining chambers that were filled with reagents used in the process. The used reagents were xylene (XYL), absolute alcohol (ABS), 94% alcohol, 70% alcohol and distilled water. Slides were put on the sledge, which was then moved from one chamber to another (figure 5). Slides were allowed to stay in distilled water containing chamber until the immunostaining was started.

FIGURE 5. The arrangement of the deparaffinisation process.

The expression of endosialin was investigated by immunohistochemical staining. Immunohisctochemical staining was performed using a staining kit (Power Vision+™ Histostaining Kit; ImmunoVision Technologies, Corporation, Brisbane, CA, USA) that contained primary antibody diluent, post-antibody blocking (post block), poly-HRP anti-mouse/rabbit igG (PV-HRP) and diaminobenzidine (DAB) solutions A and B. Two different monoclonal anti-mouse IgG primary antibodies against endosialin were used to detect endosialin in the tissue specimens. Endosialin monoclonal antibodies II-50/3 and VIII-16 were kindly provided by Dr. Silvia Pastorekova and Professor Jaromir Pastorek, Slovak Academy of Sciences, Bratislava, Slovak Republic. Production of endosialin monoclonal antibodies have been described in Ohradanova et al. 2008, 1349.

Staining was performed according to the manufacturer's protocol using fully computer operated automatic staining device, autostainer (model: 480; Lab Vision Products, Thermo Fisher Scientific, Runcorn, Cheshire, United Kingdom). Staining program was created with computer (table 1 and appendix 3, figure 2). Dispense volume of reagents was adjusted to 100µl. When the staining program was approved the computer reported the needed volumes of reagents, distilled water and Tris buffered saline with tween (TBS-Tween) used in immunostaining. Reagents used in staining process are represented in a form of reagent layout map (appendix 3, figure 3).

Reagents were put to autostainer tubes according to the reagent lay out map. Endosialin monoclonal antibody II-50/3 was diluted 1:5 in primary antibody diluent. Endosialin monoclonal antibody VIII-16 was undiluted. Slides were transferred from staining chamber to autostainer racks and moistened with TBS-Tween buffer. When prime pumps for distilled water and TBS-Tween buffer were done the autostainer was ready to start the programmed staining process. Between reagent applications staining waste was rinsed from slides either with distilled water or TBS-Tween buffer. When staining process was ready the slides were transferred back to distilled water containing staining chamber.

TABLE 1. Immunostaining program for detection of endosialin by indirect immunostaining and enzyme-labeled antibodies.

Reagent	Function is to	Time (min)	Dilution
H_2O_2	block endogeneous peroxidase	5	3%
Colostrum	block non-specific binding sites	10	1:5
Primary antibody	bind to epitope of antigen	30	1:5 / –
Post block	block mouse immunoglobulins	20	–
Secondary antibody	bind to the primary antibody	30	–
DAB	form a brown precipitate	5	1:30
$CuSO_4$	strengthen and brighten up reaction	5	0,5%
HEMA	nuclear counterstain	1	1:3

Dehydration process started from distilled water containing staining chamber. Reagents were same as were used in deparaffinisation process but in reverse order. The sledge containing slides was transferred from one chamber to another (figure 6). Cover glasses

were applied on stained tissue sections. A mounting medium (Pertex, xylene-based; Histolab products AB, Göteborg, Sweden) is used to attach cover glasses. Few drops of mounting medium were pipetted onto the tissue sections and cover glasses were placed onto tissue. Air bubbles were removed by gently pressing the cover glass against tissue sections. Slides were allowed to dry before microscopic analysis.

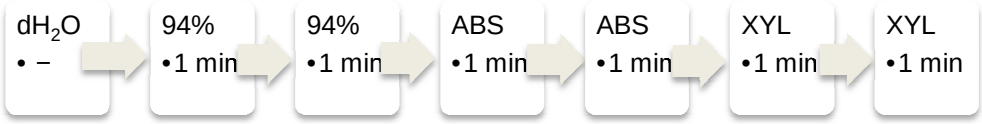


FIGURE 6. The arrangement of dehydration process.

4.4 Microscopic and statistical analysis

The microscopic analysis of endosialin in immunostained tissue sections was performed with a light microscope with professional help of Professor Seppo Parkkila and Hannu Haapasalo, MD, PhD, from department of pathology, Tampere university hospital. In microscopic analysis the idea was to evaluate the intensity of immunostaining reaction separately with normal and cancerous tissue on a scale of 0 to 3. Thus every immunostained specimen was categorized to one of the four groups where 0 indicated that no reaction was detectable, 1 was for weak staining, 2 for medium strength staining and 3 for intense staining.

In analysis the structure of tissues was first examined to identify normal and cancerous sections in samples. Then both were categorized according to the intensity of immunostaining reaction. Depending on tissue type either endothelium or epithelium was analyzed. In addition, in nervous tissue also blood vessels were analyzed.

Data was analyzed statistically with GraphPad Prism software. Correlation between endosialin antibodies was studied with Spearman correlation. Staining intensity between different tumor types was analyzed with unpaired t-test where p-value 0,05 was considered statistically significant. Staining intensity between different tumor grades was studied with one-way analysis of variance where p-value 0,05 was considered statistically significant.

5 RESULTS

5.1 Function of novel endosialin monoclonal antibodies

Both endosialin monoclonal antibodies II-50/3 and VIII-16 showed good expression in stained tissue specimens. The used staining protocol and dilutions under both endosialin antibodies were suitable. It appeared that there was variation in staining reactivity rather between tissue types than between antibodies. Antibody II-50/3 generally showed slightly weaker staining reactivity. Staining intensity was clearly stronger in brain tumors and in skin specimens than it was in colorectal cancer, pancreatic cancer or in hepatobiliary lesions. Background staining was overall insignificant and nuclei counterstaining was successful.

It became evident that staining reactivity was generally lower in normal tissues compared to cancerous tissues with both antibodies. This result was the most clearly visible in nervous tissues of the brain specimens as shown in figures 9 and 10, where brain tumors showed clearly stronger staining intensity than normal brain. There were some differences in staining reactivity between antibodies II-50/3 and VIII-16. Interestingly for example in normal skin antibody VIII-16 showed stronger intensity but in normal colon the intensity was vice versa (figures 7A and B). There was medium correlation in staining reactivity within two antibodies in normal skin and in normal colon tissues. Staining results in normal skin showed not statistically significant medium correlation between antibodies (Spearman r^2=0,569, p=0,200) (figure 7A). Staining results in normal colon showed highly statistically significant medium correlation between antibodies (Spearman r^2=0,493, p=0,0007) (figure 7B).

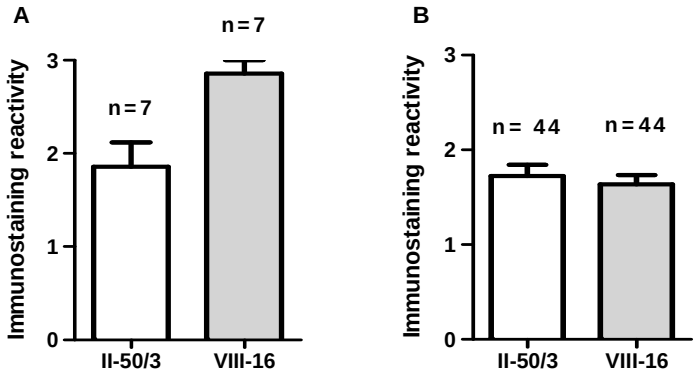


FIGURE 7. Expression of endosialin in normal tissues. A. Normal skin and B. Normal colon.

5.2 Expression of endosialin in tumors

5.2.1 Endosialin in brain tumors

Immunostaining reaction in brain tumor specimens was overall quite intense (figures 9 and 10). Positive reaction was detectable in both nervous tissues and blood vessels of the specimens. It showed that staining intensity was generally stronger in blood vessels than in nervous tissues with either of the endosialin antibodies. Moreover staining intensity was the strongest in blood vessels stained with antibody VIII-16 (figure 10D).

Mean intensity of blood vessels stained with antibody II-50/3 varied between 1,2 ± 0,1 (SEM) – 2,0 ± 0,0 (SEM) (standard error of mean) with different types of brain tumors (figure 8B). Mean intensity of blood vessels stained with antibody VIII-16 varied between 1,9 ± 0,2 (SEM) – 2,3 ± 0,3 (SEM) with different types of brain tumors (figure 8D). In every occasions the most intense staining could be seen with gliosarcoma, but the case number (n=3) may have had an effect on the result (figure 8).

There were two statistically significant differences in endosialin expression between different brain tumor types. These differences were observed with antibody VIII-16 stained nervous tissues. The difference in endosialin expression between glioblastoma multiforme and gliosarcoma was statistically significant (p=0,04, unpaired t-test). Other statistically significant difference (p=0,02, unpaired t-test) in endosialin expression was

observed between diffuse astrocytoma and gliosarcoma. No statistically significant difference in endosialin expression (p>0,05, unpaired t-test) was observed between nervous tissues or blood vessels stained with antibody II-50/3 and blood vessels stained with antibody VIII-16.

Both nervous tissue and blood vessels showed the most intense immunostaining reaction with both endosialin antibodies under anaplastic astrocytoma, grade 3. However no statistically significant difference (p>0,05, one-way analysis of variance) was observed in the endosialin immunostaining reactions between different tumors grades, data not shown.

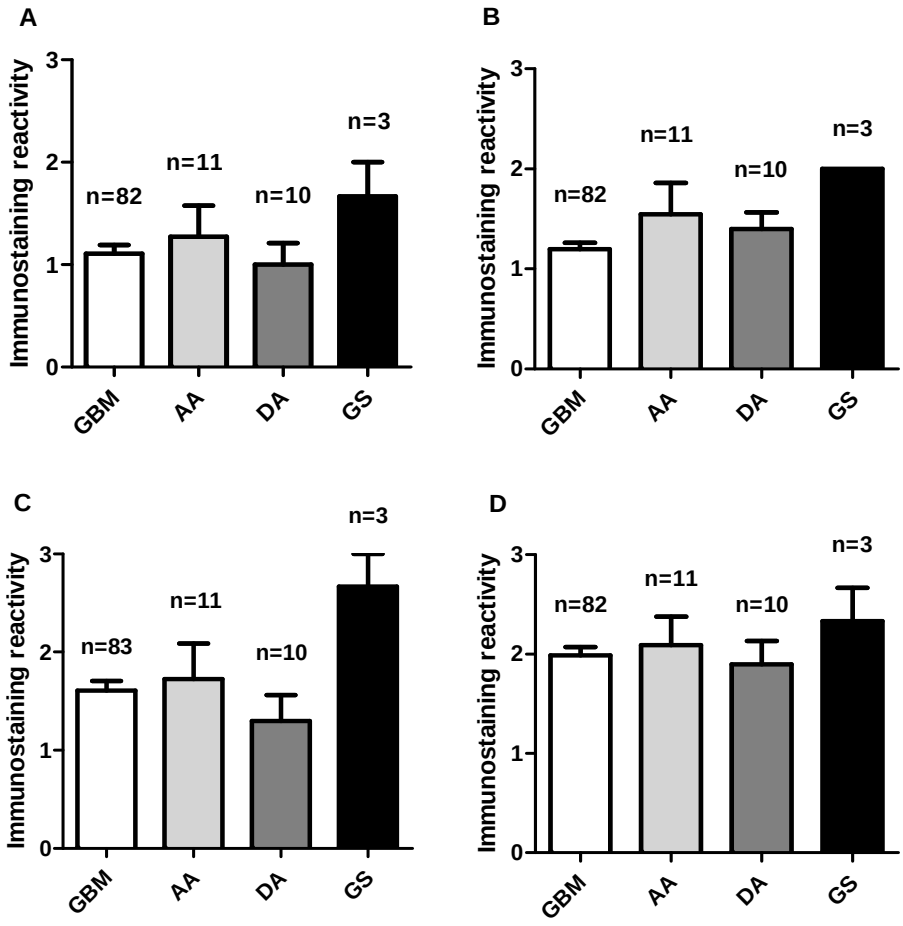


FIGURE 8. Immunostaining reactivity in brain tumors. A. Nervous tissue stained with antibody II-50/3, B. Blood vessels stained with antibody II-50/3, C. Nervous tissue stained with antibody VIII-16 and D. Blood vessels stained with antibody VIII-16. GBM = Glioblastooma multiforme, AA = Anaplastic astrocytoma, DA = Diffuse astrocytoma and GS = Gliosarcoma.

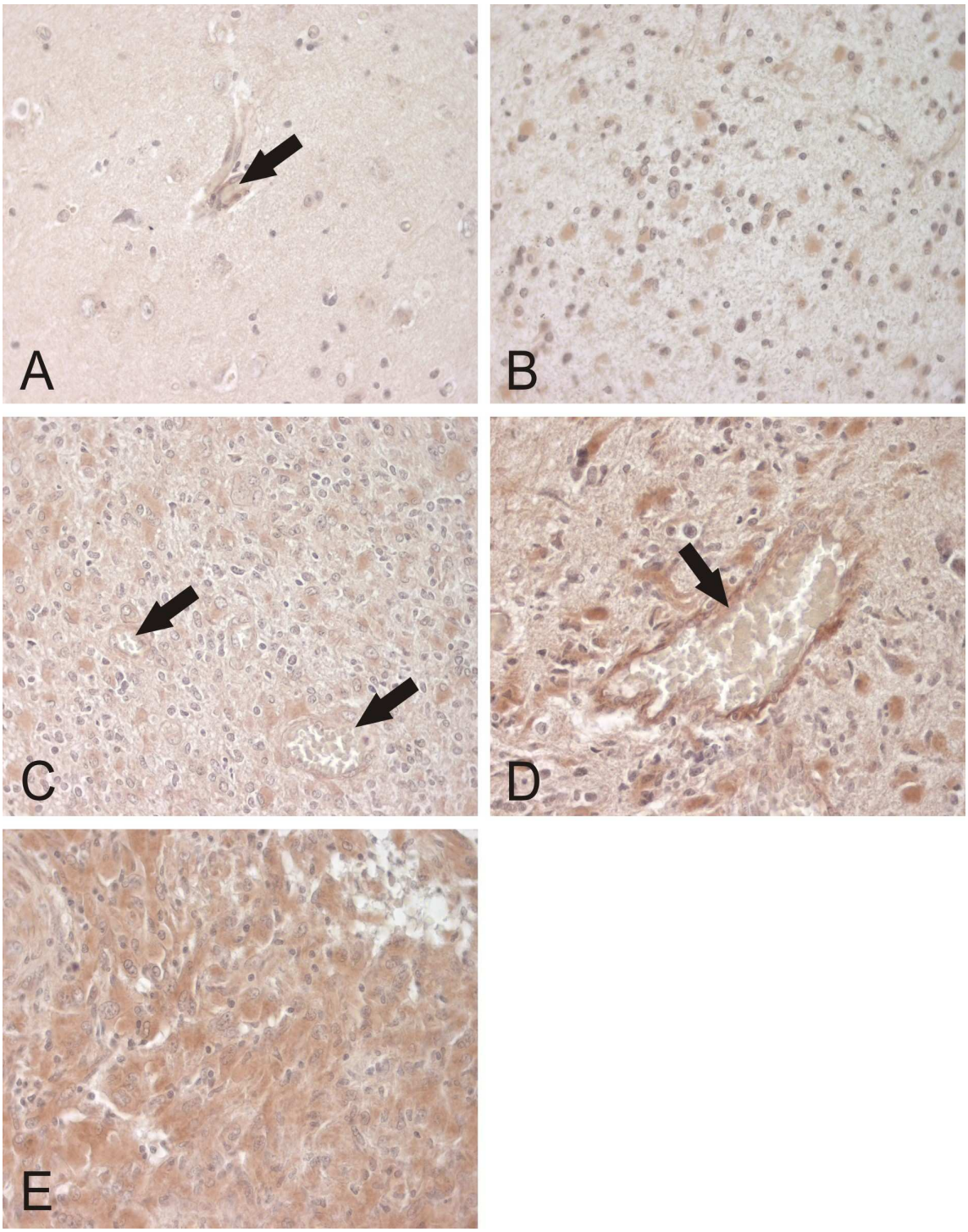


FIGURE 9. Endosialin in brain tumors, stained with endosialin antibody II-50/3. A. Normal brain, B. Anaplastic astrocytoma, C. and D. Glioblastoma multiforme and E. Gliosarcoma. Arrows represent blood vessels.

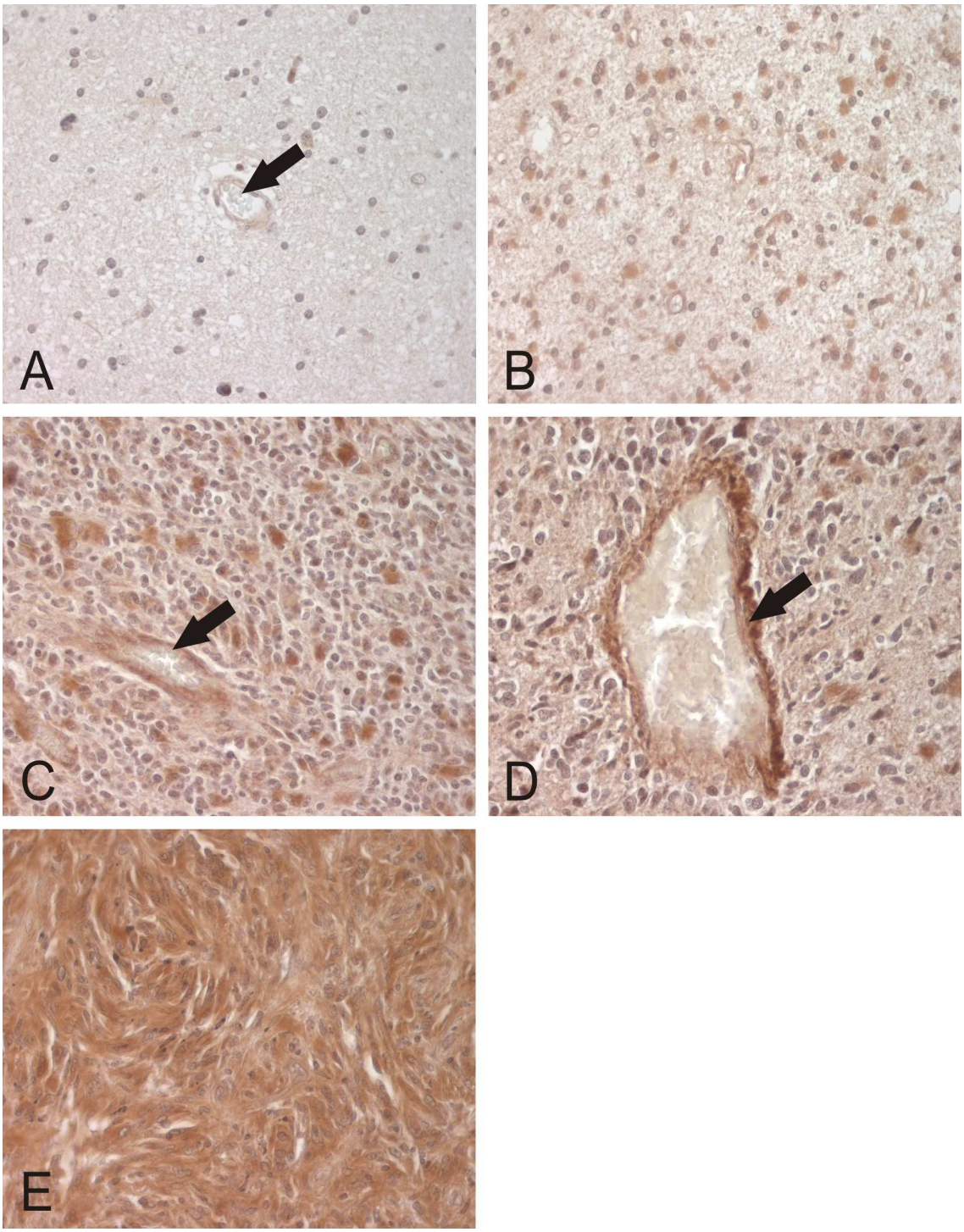


FIGURE 10. Endosialin in brain tumors, stained with endosialin antibody VIII-16. A. Normal brain, B. Anaplastic astrocytoma, C. and D. Glioblastoma multiforme and E. Gliosarcoma. Arrows represent blood vessels.

5.2.2 Endosialin in colorectal cancers

There was lots of variation in immunostaining intensity in colorectal cancer specimens. Sometimes normal colon showed very intense staining but elsewhere staining intensity was negative. All colorectal cancers behaved similarly. For example a comparison between two normal colon specimens showed both weak and very intense staining as shown in figures 13A and B and 14A and B. On the other hand a comparison between colorectal cancers microsatellite stable (MSS) and gastrointestinal stromal tumor (GIST) showed same kind of result as shown in figures 13E and G and 14E and G. Interestingly it was also noticed that human normal colon smooth muscle staining was very intense with both endosialin antibodies (figures 13C and 14C).

Comparison of immunostaining reactivity between colorectal cancers showed the most intense staining with both endosialin antibodies in microsatellite instable (MSI) specimens (figures 11A and B). Mean immunostaining intensity of MSI with antibody II-50/3 varied between 1,533 ± 0,2 (SEM). Mean immunostaining intensity of MSI with antibody VIII-16 varied between 1,688 ± 0,2 (SEM). These results indicated that overall immunostaining intensity was again slightly more intense with antibody VIII-16.

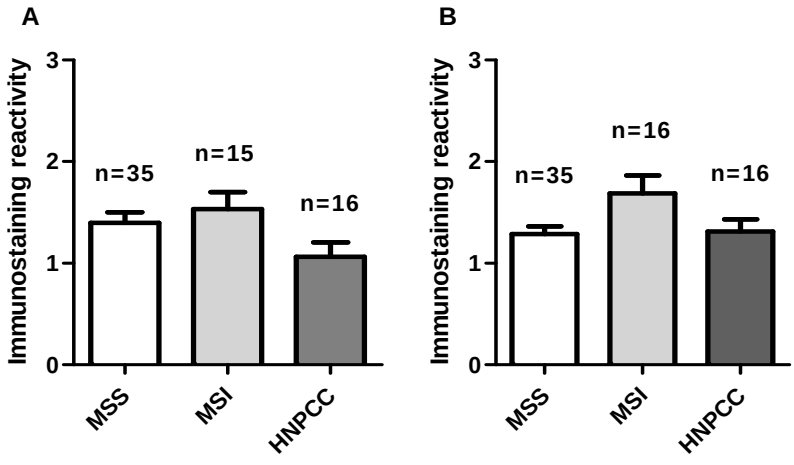


FIGURE 11. Immunostaining reactivity in colorectal cancers. A. Stained with antibody II-50/3 and B. Stained with antibody VIII-16. MSS = Microsatellite stable, MSI = Microsatellite instable and HNPCC = Hereditary nonpolyposis colorectal cancer.

There were three statistically significant differences in endosialin expression between different colorectal cancer types. These differences were found with both endosialin

antibodies. The differences in endosialin expression between MSS and MSI were statistically significant with both antibodies (II-50/3: p=0,02, unpaired t-test and VIII-16: p=0,02, unpaired t-test). In addition the difference in endosialin expression between MSI and hereditary nonpolyposis colorectal cancer (HNPCC) was statistically significant (p=0,04, unpaired t-test) with antibody II-50/3.

Both endosialin antibodies showed the most intense immunostaining reaction in colorectal cancers that are grade 2 (figure 12). In the colorectal cancer material there were grade 2 cases from every colorectal cancer group MSS, MSI and HNPCC. However no statistically significant difference (p>0,05, one-way analysis of variance) was found in the endosialin immunostaining reactions between different tumor grades.

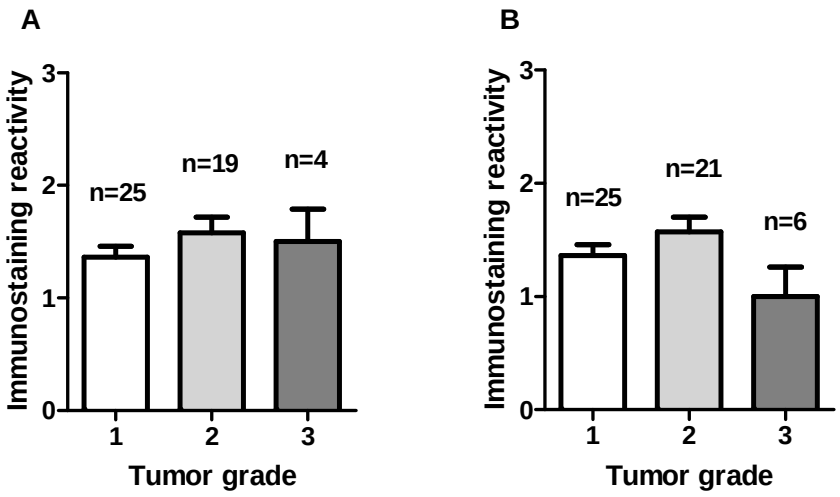


FIGURE 12. Immunostaining reactivity in different tumor grades of colorectal cancers. A. Stained with antibody II-50/3 and B. Stained with antibody VIII-16.

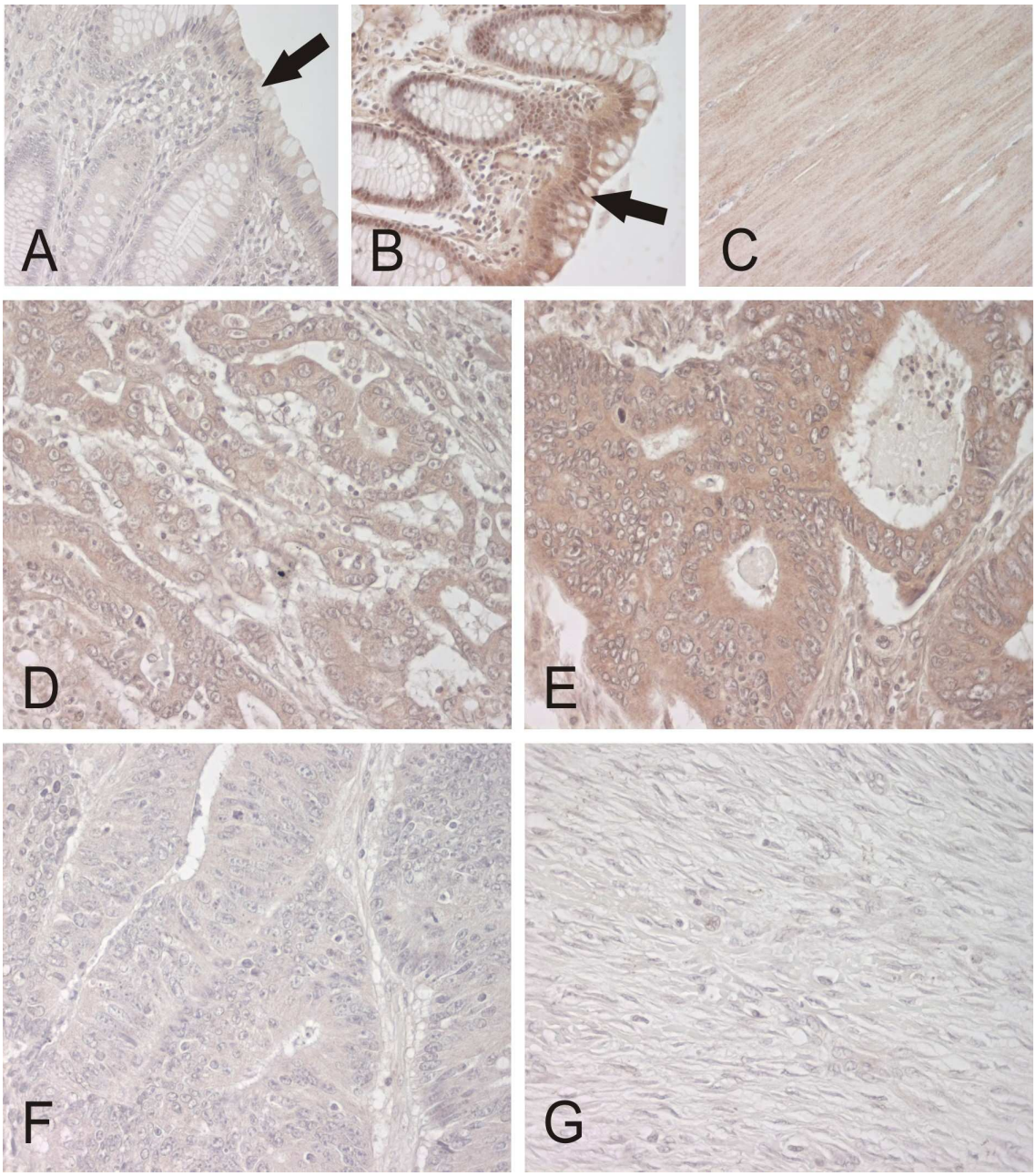


FIGURE 13. Endosialin in colorectal cancers, stained with endosialin antibody II-50/3. A. and B. Normal colon, C. Smooth muscle, D. Microsatellite instable, E. Microsatellite stable, F. Hereditary nonpolyposis colorectal cancer and G. Gastrointestinal stromal tumor. Arrows represent surface epithelial cells.

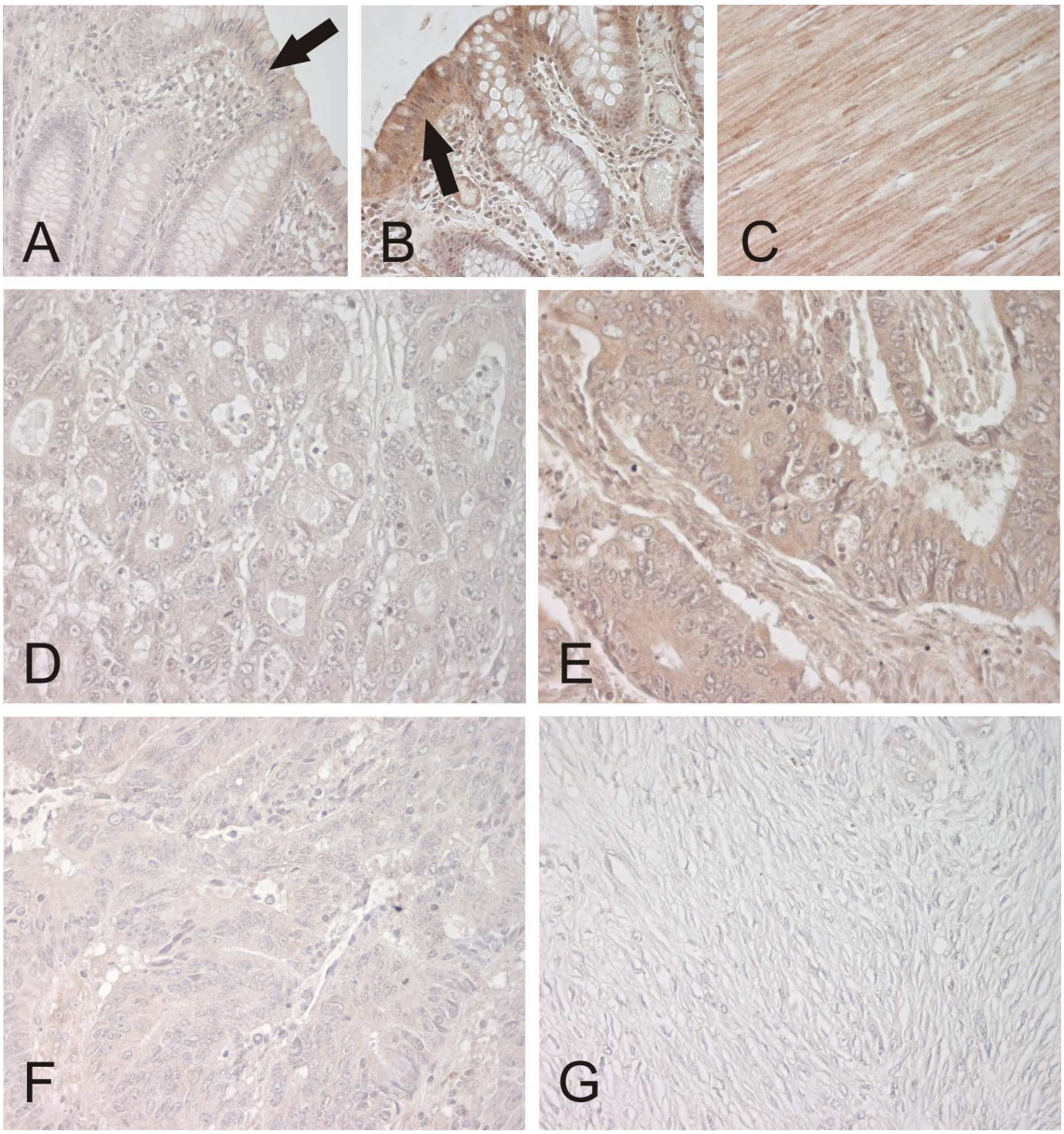


FIGURE 14. Endosialin in colorectal cancers, stained with endosialin antibody VIII-16. A. and B. Normal colon, C. Smooth muscle, D. Microsatellite instable, E. Microsatellite stable, F. Hereditary nonpolyposis colorectal cancer and G. Gastrointestinal stromal tumor. Arrows represent surface epithelial cells.

5.2.3 Endosialin in pancreatic cancers

Pancreatic specimens stained with endosialin antibodies behaved in an interesting way. Firstly, there was not a visible difference in staining intensity between normal pancreatic tissue and cancerous pancreas stained with either of the antibodies as shown in figures 16A, C and E and 16B, D and F. Secondly, it was observed that pancreatic ducts stained very intensively in normal pancreas specimens with either of the endosialin antibodies (figures 16A and B). The same phenomenon was seen with pancreatic cancer specimens (figures 16C and D). However the overall immunostaining intensity was not particularly strong in pancreas specimens.

There were not statistically significant differences in immunostaining intensity between pancreatic cancers and pancreatic ducts, ($p>0,05$, unpaired t-test). Statistically the intensity of staining in pancreatic cancers was more intense with antibody VIII-16 (figures 15A and B). (II-50/3: 0,7 ± 0,2 (SEM) and VIII-16: 1,1 ± 0,2 (SEM), unpaired t-test). However the staining intensity of pancreatic ducts was more intense with antibody II-50/3 (figures 15A and B). (II-50/3: 1,5 ± 0,5 (SEM) and VIII-16: 1,3 ± 0,3 (SEM), unpaired t-test).

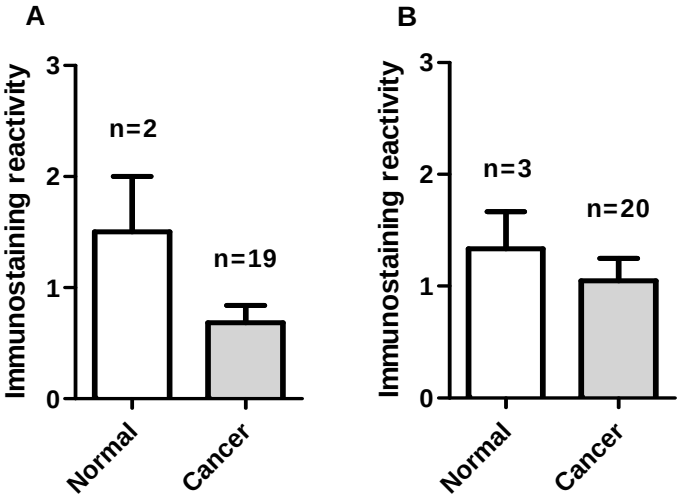


FIGURE 15. Immunostaining reactivity in pancreas specimens. A. Stained with antibody II-50/3 and B. Stained with antibody VIII-16.

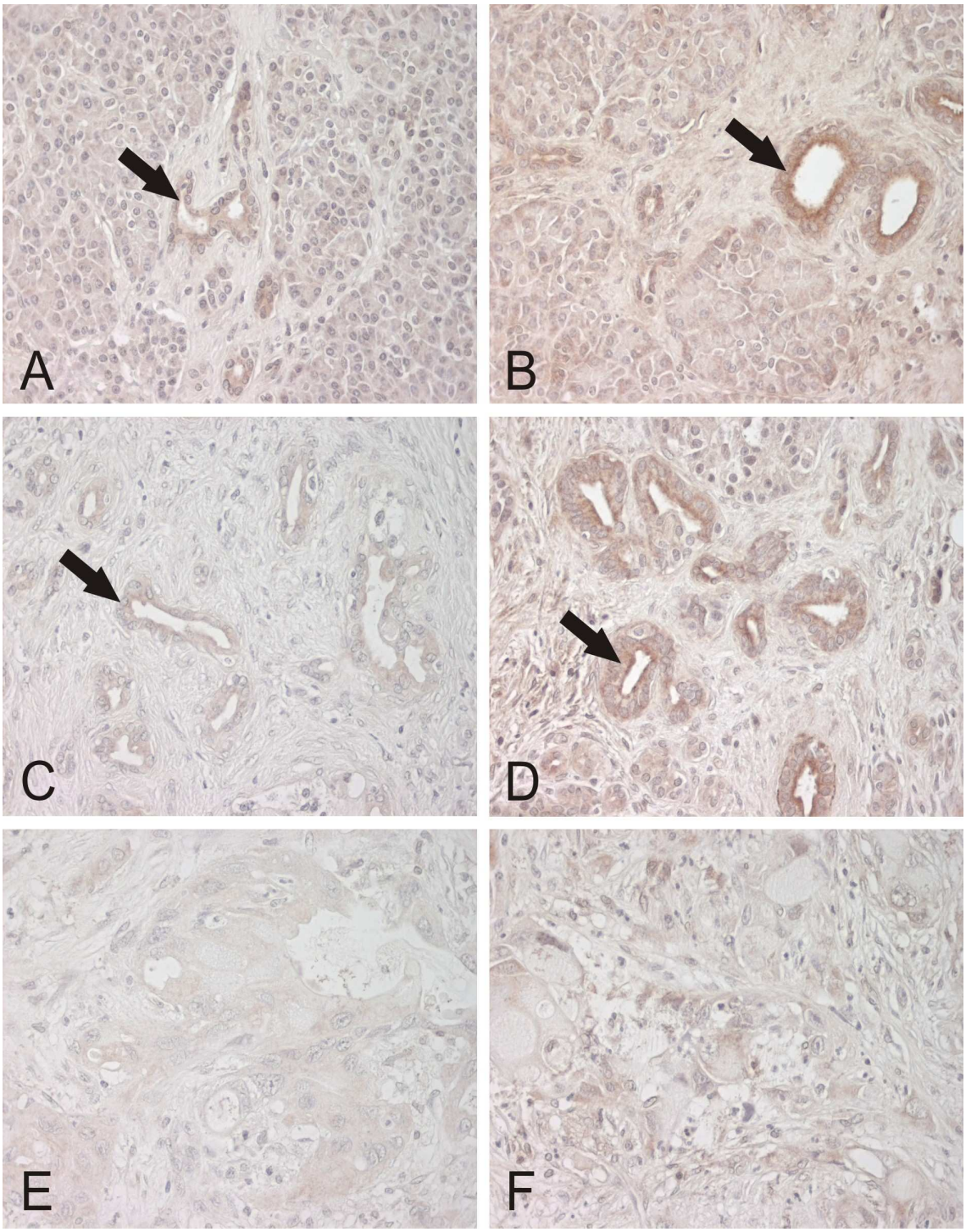


FIGURE 16. Endosialin in pancreatic cancers, stained with endosialin antibodies II-50/3 and VIII-16. A. Normal pancreas stained with II-50/3, B. Normal pancreas stained with VIII-16, C. Pancreatitis chronica stained with II-50/3, D. Pancreatitis chronica stained with VIII-16, E. Adenocarcinoma ductale stained with II-50/3 and F. Adenocarcinoma ductale stained with VIII-16. Arrows represents pancreatic ducts.

5.2.4 Endosialin in hepatobiliary lesions

There was no detectable positivity in immunostaining reaction in liver specimens stained with either of the endosialin antibodies as shown in figures 17A and B. In addition, all hepatobiliary lesions as well as normal liver specimens proved to be negative with either of the endosialin antibodies (figures 17E and F and 17G and H).

The sample material of liver included, besides normal and cancerous specimens, hepatic-, cirrhotic- and fatty liver specimens (figures 17C and D). Data of hepatic- and cirrhotic specimens are not shown. All of these specimens tested negative in immunostaining with either of the endosialin antibodies. It can be stated that neither of antibodies functioned better in hepatobiliary lesions. In this situation statistical analysis of hepatobiliary lesions would prove unnecessary.

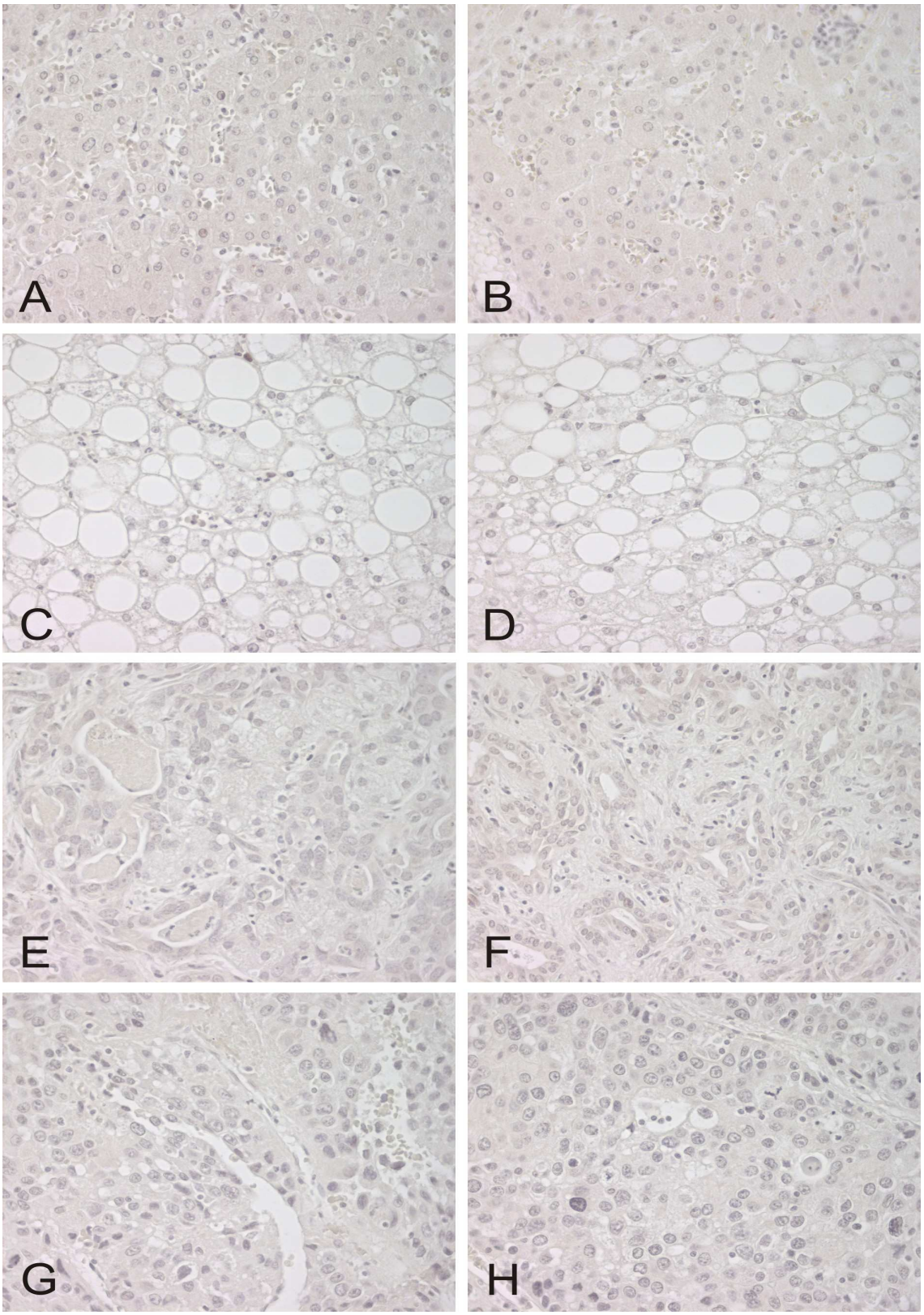


FIGURE 17. A. Normal liver stained with II-50/3, B. Normal liver stained with VIII-16, C. Fatty liver stained with II-50/3, D. Fatty liver stained with VIII-16, E. Colangio carcinoma stained with II-50/3, F. Colangio carcinoma stained with VIII-16, G. Hepatocellular carcinoma stained with II-50/3 and H. Hepatocellular carcinoma stained with VIII-16.

5.2.5 Endosialin in skin tumors

Immunostaining reaction was very intense in skin specimens. Positive reaction occurred in both epidermis and dermis of normal skin specimens. Epidermis showed very intense reaction and dermis medium strength reaction with either of the endosialin antibodies (figures 19A and B). Skin cancers also showed very intense positive reaction, although with antibody VIII-16 reaction seemed to be a bit stronger (figures 19C and D). It was observed that there was variation in staining intensity rather between antibodies than between normal skin and skin cancers (figures 18A and B).

There were not statistically significant difference in immunostaining reactivity between normal skin and skin cancers, ($p>0,05$, unpaired t-test). As it was already observed the variation in immunostaining intensity occurred between antibodies. This can be proved statistically as well. With antibody II-50/3 staining intensity between normal skin and skin cancers was almost similar (1,9 ± 0,3 (SEM) and 2,0 ± 0,3 (SEM), unpaired t-test) (figure 18A). With antibody VIII-16 same phenomenon was seen between normal skin and skin cancers (2,9 ± 0,1 (SEM) and 2,8 ± 0,3 (SEM), unpaired t-test) (figure 18B).

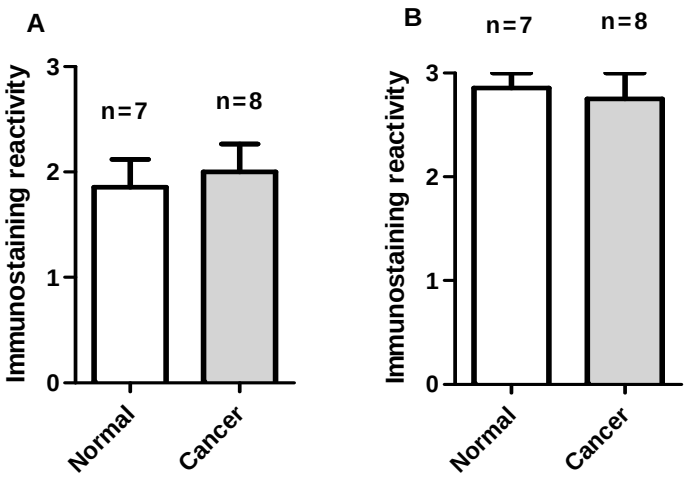


FIGURE 18. Immunostaining reactivity in normal skin and in skin cancers. A. Stained with antibody II-50/3 and B. Stained with antibody VIII-16.

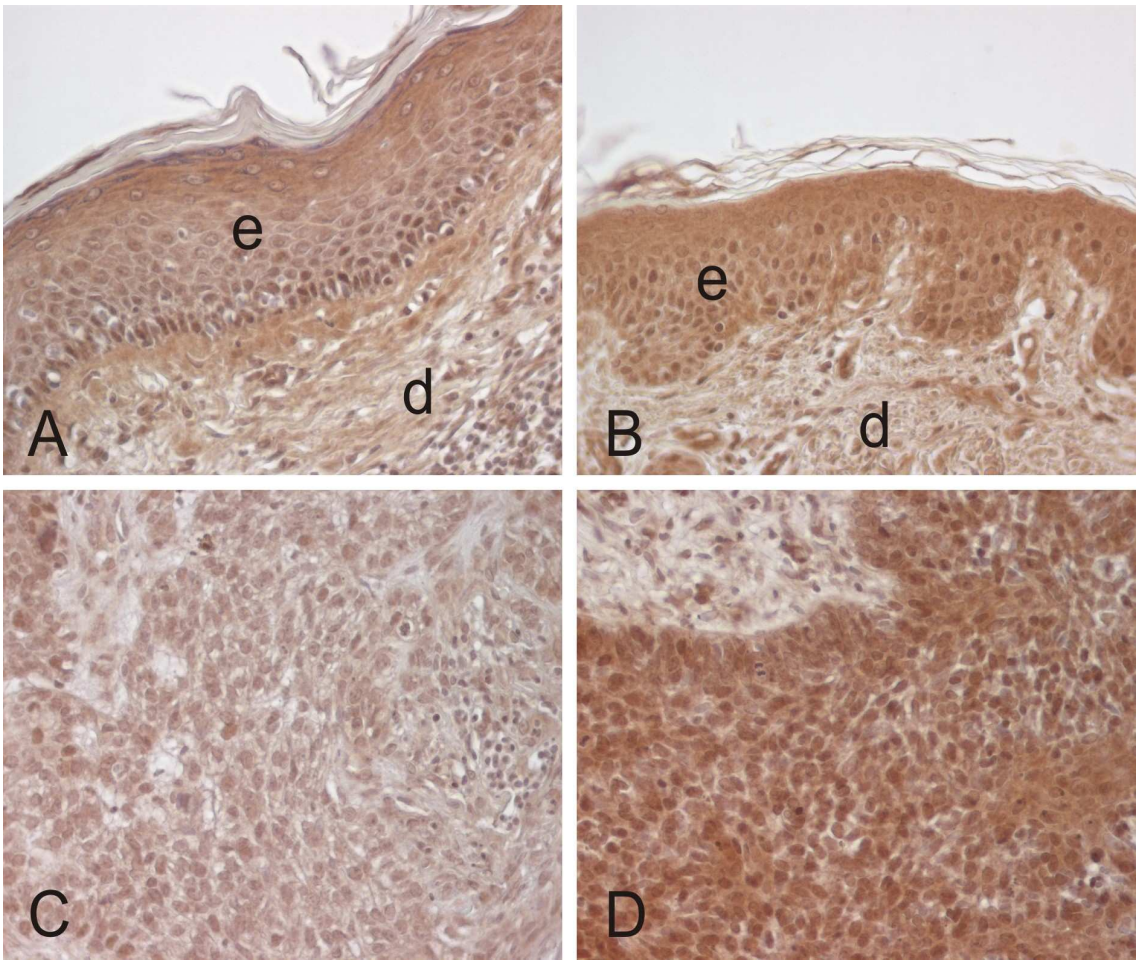


FIGURE 19. Endosialin in skin stained with antibodies II-50/3 and VIII-16. A. Normal skin stained with II-50/3, B. Normal skin stained with VIII-16, C. Basocellular carcinoma stained with II-50/3 and D. Basocellular carcinoma stained with VIII-16. In figures e represents epidermis and d represents dermis.

6 DISCUSSION

The aim of this study was to investigate expression of endosialin in tumor specimens by immunohistochemistry. Thin tissue sections were produced from tissue blocks with microtome. Tissue specimens were immunostained with automatic staining device using a staining kit. Immunostaining was performed according to the manufacturer's protocol using indirect immunostaining technique. The secondary antibody labeled with HRP-enzyme label was able to recognize endosialin found in tissue specimens. The formed enzyme reaction was visible for naked eyes and thus suitable for light microscopic examination. Intensity of immunostaining reaction was studied and results were analyzed statistically.

It was found that both of the novel endosialin monoclonal antibodies II-50/3 and VIII-16 functioned well in immunostaining. The used staining protocol appeared to be suitable for detection of endosialin. In addition, dilutions under both endosialin monoclonal antibodies were found to be adequate, because there was not considerable variation in staining intensity between antibodies. Automatic staining decreases the possibility of faults that might happen in manual staining.

However it was observed that the overall staining intensity was slightly more intense with endosialin monoclonal antibody VIII-16 compared to the antibody II-50/3. This phenomenon is seen in normal tissues as well as in cancerous specimens with the exception of normal colon specimens. In normal colon specimens the staining intensity with the antibody II-50/3 proved to be bit more intense compared to the staining intensity with the antibody VIII-16.

It became evident that staining intensity was generally lower in normal tissues compared to cancerous tissues stained with either of the endosialin antibodies. This result was the most clearly visible in nervous tissues of the brain specimens where brain tumors showed clearly stronger intensity than normal nervous tissue of brain specimens. This was an expected result and confirms the theory that endosialin is expressed more intensively in cancerous tissues than in normal tissues.

There were differences in staining intensity between tissue types. Generally staining intensity was more intense in brain-, colon- and skin tumors compared to pancreatic cancers and hepatobiliary lesions. In pancreatic specimens the pancreatic ducts were observed to stain more intensively than normal pancreas or pancreatic cancer specimens. In addition, every liver specimen proved to be negative despite the fact that the sample material contained besides normal and cancerous tissues also cirrhotic- and fatty liver specimens.

There was a lot of variation in staining intensity in colon specimens. There were colorectal cancers that stained on the other hand very intensively with either of the endosialin antibodies, but elsewhere the staining intensity was nearly negative. Same phenomenon was seen as well in normal colon specimens. In conclusion any specificity to expression of endosialin in colorectal cancers was not found compared to normal colon specimens.

After all, the most intense staining was detected in skin specimens stained with the endosialin antibody VIII-16. However it was found that normal skin specimens stained more intensively than skin cancer specimens (2,9 ± 0,1 (SEM) and 2,8 ± 0,3 (SEM), unpaired t-test). The gained staining result is contrary to expected result. It became evident, that expression of endosialin in skin specimens showed not specificity to cancers.

On the contrary to skin specimens the expression of endosialin in brain tumors showed some specificity to cancerous tissues. Intense staining reactivity was not found in normal brain tissues, but both nervous tissues and blood vessels of brain tumors stained intensively. The most intense staining reactivity in brain tumors was seen in blood vessels stained with endosialin antibody VIII-16. The mean intensity varied between 1,9 ± 0,2 (SEM) – 2,3 ± 0,3 (SEM) unpaired t-test).

Regardless of the good staining results statistic correlation between staining intensity and the tumor grade was not found either in brain tumors or in colorectal cancer specimens. The results of this study did not clearly indicate whether endosialin could function as a specific molecular biomarker. In other words according to this study it cannot be confirmed that increased concentration levels of endosialin indicate cancer. However according to the results of this study it appears that the most potential targets for future

research of endosialin are brain tumors. Brain tumor research may have potential towards the development to specify the main function of endosialin.

There are various studies in which expression of endosialin has been studied by immunohistochemistry. The expression of endosialin has been demonstrated in lots of human tumor tissue types in different studies. There are results that show similarity to the results of this study. In the immunohistochemical study of human brain tumors there were expression of endosialin in brain tumor specimens. Normal brain tissues were negative and did not express endosialin (Brady et al. 2004, 1274, 1283). Similar results were reported with immunostained human colon carcinoma biopsies (Bagley et al. 2008, 187).

Normal tissues have been immunostained in a study of mouse endosialin expression. Endosialin was observed to be expressed during the whole embryonic stage, but not in newborns or adult mice. (Rupp et al. 2006, 1, 4.) According to the results of this study, it was found that normal tissues, especially skin- and colon tissues, expressed endosialin. There is no previous study demonstrating expression of endosialin in normal human tissues.

When it comes to ethics, this study can be considered ethical. The sample material for this study was gained from hospitals. All tumor specimens used in this study were obtained by surgeons from patients that were having symptoms because of some disease. Secondly, all of the specimens were removed in surgical procedure in order to find out histopathology of the disease and to understand the physiology of the disease. Through understanding of the histopathology and physiology of the disease it was possible to give patients the best treatment.

In this study, lots of information about the expression of endosialin was produced. The expression was studied in several types of tissues and in both normal and cancerous tissues. In consequence this study offers wide-ranging point of view. The results of this study can be considered reliable, because staining intensities were quite comparable between each tumor types and because in statistical analysis standard errors of means were quite small. Endosialin may play a part in future cancer research and in development of targeted cancer drugs.

In future it would be interesting to investigate the expression of endosialin with two different methods to see if the results show any similarity to the results of this study. The difference could be for example between labels. One of the labels could be an enzyme and the other a fluorescent. On the other hand it would be also worth trying to study expression of endosialin for example with RT-PCR to identify RNA-transcripts. The study of RNA-transcripts would bring some extra value for this study.

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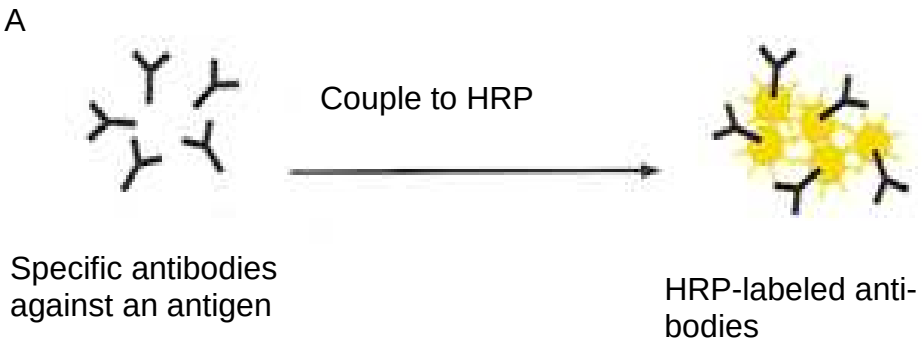
APPENDICES

APPENDIX 1

INDIRECT IMMUNOSTAINING METHOD



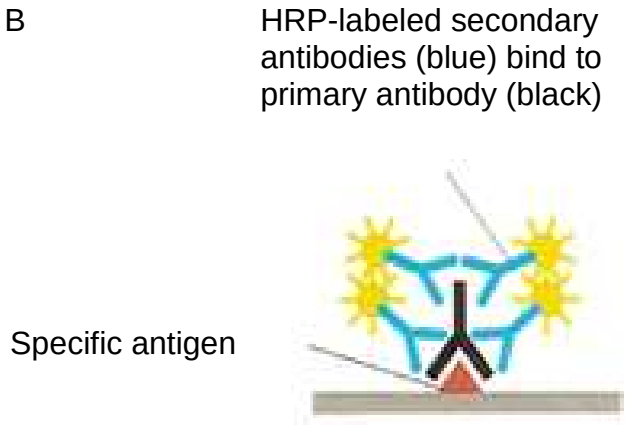


FIGURE 1. Antibody labeling and indirect immunostaining method. A. Antibodies are labeled with HRP-enzyme label. B. Illustration of indirect immunostaining method. (Modified from Alberts et al. 2009, 145)

APPENDIX 2

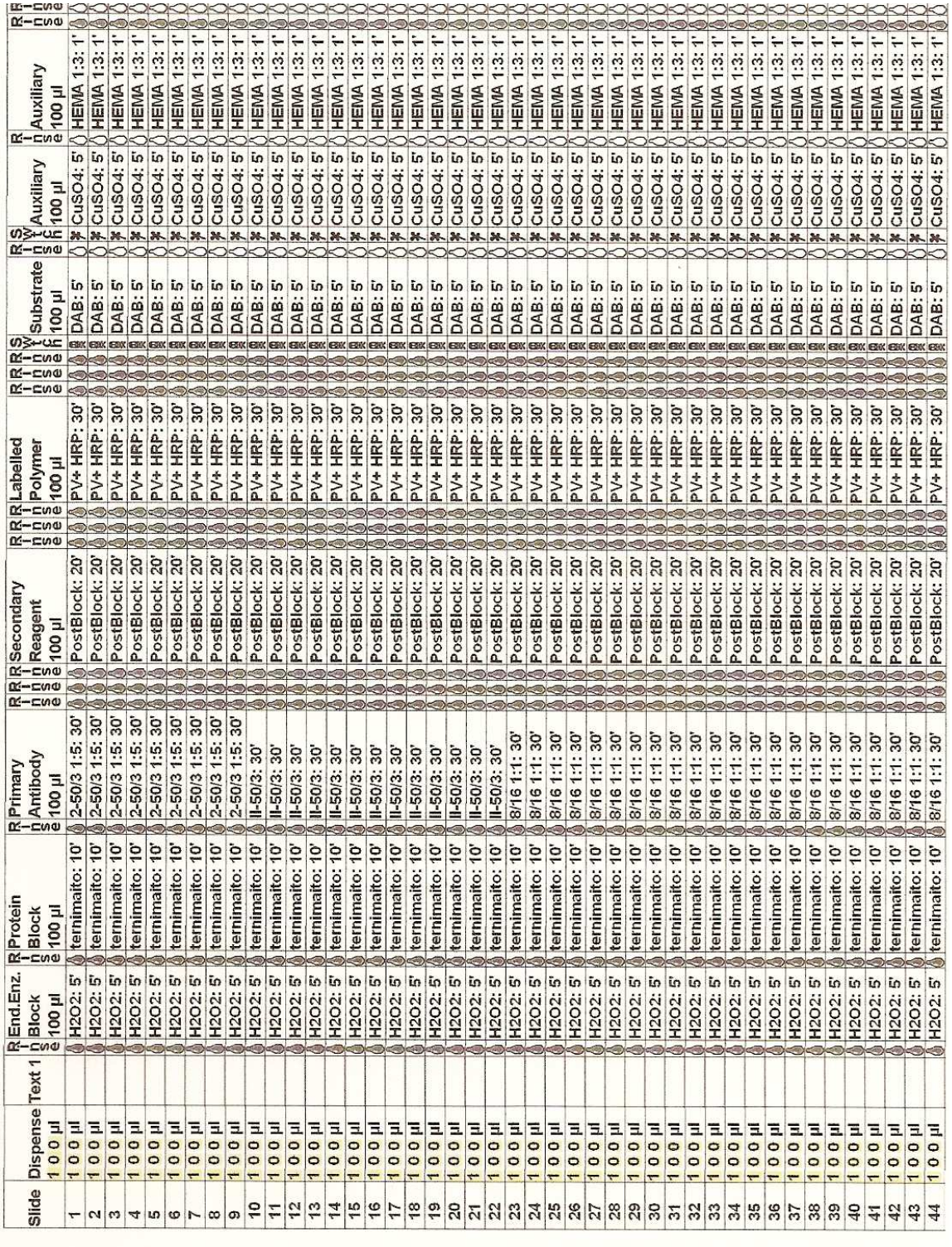
SUMMARY OF STAINED TISSUE SPECIMENS

TABLE 1. Summary of stained tissue specimens divided into categories according to tissue types and staining with different endosialin antibodies. Category others contains specimens from duodenum, esophagus, antrum, corpus, colon and rectum.

Tissue type	n (II-50/3)	n (VIII-16)
Brain:		
Normal brain:		
Nervous tissue	7	7
Blood vessels	7	7
Brain tumors:		
Nervous tissue	106	106
Blood vessels	106	106
Colon:		
Normal colon	44	44
Colorectal cancers	66	66
Pancreas:		
Normal pancreas	2	3
Pancreatic cancers	19	20
Liver:		
Normal liver	3	3
Hepatobiliary lesions	6	6
Others (fatty liver, cirrhosis and hepatitis)	9	9
Skin:		
Normal skin	6	6
Skin cancers	8	8
Other tissues:		
Normal tissues	20	20
Cancerous tissues	4	4
Total of normal tissue specimens:	197	198
Total of cancerous tissue specimens:	216	217
Total:	413	415

APPENDIX 3

IMMUNOSTAINING PROGRAM AND REAGENT LAYOUT MAP



Slide	Dispense 100 µl	Text 1	Rinse	End.Enz. Block 100 µl	Rinse	Protein Block 100 µl	Rinse	Primary Antibody 100 µl	Rinse	Rinse	Secondary Reagent 100 µl	Rinse	Rinse	Labelled Polymer 100 µl	Rinse	Rinse	Substrate 100 µl	Rinse	Rinse	Auxiliary 100 µl	Rinse	Rinse
1	100 µl			H2O2: 5'		ternimaito: 10'		2-50/3 1:5: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
2	100 µl			H2O2: 5'		ternimaito: 10'		2-50/3 1:5: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
3	100 µl			H2O2: 5'		ternimaito: 10'		2-50/3 1:5: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
4	100 µl			H2O2: 5'		ternimaito: 10'		2-50/3 1:5: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
5	100 µl			H2O2: 5'		ternimaito: 10'		2-50/3 1:5: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
6	100 µl			H2O2: 5'		ternimaito: 10'		2-50/3 1:5: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
7	100 µl			H2O2: 5'		ternimaito: 10'		2-50/3 1:5: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
8	100 µl			H2O2: 5'		ternimaito: 10'		2-50/3 1:5: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
9	100 µl			H2O2: 5'		ternimaito: 10'		2-50/3 1:5: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
10	100 µl			H2O2: 5'		ternimaito: 10'		II-50/3: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
11	100 µl			H2O2: 5'		ternimaito: 10'		II-50/3: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
12	100 µl			H2O2: 5'		ternimaito: 10'		II-50/3: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
13	100 µl			H2O2: 5'		ternimaito: 10'		II-50/3: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
14	100 µl			H2O2: 5'		ternimaito: 10'		II-50/3: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
15	100 µl			H2O2: 5'		ternimaito: 10'		II-50/3: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
16	100 µl			H2O2: 5'		ternimaito: 10'		II-50/3: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
17	100 µl			H2O2: 5'		ternimaito: 10'		II-50/3: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
18	100 µl			H2O2: 5'		ternimaito: 10'		II-50/3: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
19	100 µl			H2O2: 5'		ternimaito: 10'		II-50/3: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
20	100 µl			H2O2: 5'		ternimaito: 10'		II-50/3: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
21	100 µl			H2O2: 5'		ternimaito: 10'		II-50/3: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
22	100 µl			H2O2: 5'		ternimaito: 10'		II-50/3: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
23	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
24	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
25	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
26	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
27	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
28	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
29	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
30	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
31	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
32	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
33	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
34	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
35	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
36	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
37	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
38	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
39	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
40	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
41	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
42	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
43	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	
44	100 µl			H2O2: 5'		ternimaito: 10'		8/16 1:1: 30'			PostBlock: 20'			PV+ HRP: 30'			DAB: 5'			CuSO4: 5'			HEMA 1:3: 1'	

FIGURE 2. Immunostaining program. This staining program is an example of program which is suitable for HRP-labeled indirect immunostaining. The primary antibody is the only variable in the program. Grey drops represent buffer rinse and white drops distilled water rinse. Incubation time is shown after each reagent.

(continues)

APPENDIX 3:2 (2)

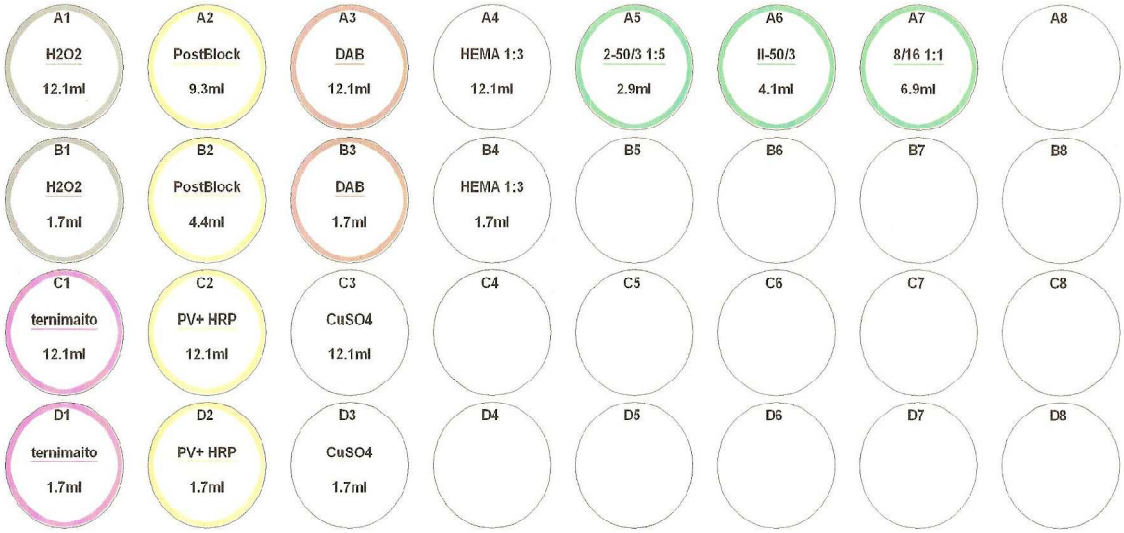


FIGURE 3. Reagent layout map for immunostaining. The reagent layout map is based on the staining program. It represents the autostainer tube rack and reports for the volumes of reagents needed in immunostaining. Reagent volumes vary according to the number of slides to be stained.