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DISSERTATION

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Expression of Cell Cycle Regulators and Melatonin Receptor 1 in Human Breast Cancer Tissue: Relevance for Therapy

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Verfasserin:	Mag. pharm. Olga RÖGELSPERGER
Matrikel-Nummer:	9601223
Dissertationsgebiet :	Pharmazie (A 091 449)
Betreuer:	Ao. Univ.-Prof. Mag. Dr. Walter JÄGER

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ABBREVIATIONS

BSA	**B**ovine **S**erum **A**lbumin
Ca	**Ca**rcinoma (Tissue)
cAMP	**c**yclic **A**denosine **M**ono**p**hosphate
CDK	**C**yclin **D**ependent **K**inase
CK	**C**yto**k**eratin
CKI	**C**yclin Dependent **K**inase **I**nhibitor
CISH	**C**hromogenic ***in situ*** **H**ybridisation
CNS	**C**entral **N**ervous **S**ystem
CRE	**c**AMP **R**esponsive **E**lement
CREB	**c**AMP **R**esponsive **E**lement **B**inding **P**rotein
CYP	**Cy**tochrome **P**450
CSC	**C**ancer **S**tem **C**ell
DAG	**D**i**a**cyl**g**lycerol
DCIS	**D**uctal **C**arcinoma **i**n **s**itu
DFS	**D**isease **F**ree **S**urvival
E2	Estradiol
EGFR	**E**pidermal **G**rowth **F**actor **R**eceptor
ER	**E**strogen **R**eceptor
ERE	**E**strogen **R**esponsive **E**lement
FISH	**F**luorescence ***in situ*** **H**ybridisation
GF	**G**rowth **F**actor
GPCR	**G**-**P**rotein **C**oupled **R**eceptor
HER2	**H**uman **E**pidermal Growth Factor **R**eceptor **2**

HPF	**H**igh **P**ower **F**ield
Id	**i**nvasive **d**uctal
IF	**I**ntermediate **F**ilament
Ig	**I**mmuno**g**lobulin
IHC	**I**mmuno**h**isto**c**hemistry
Il	**i**nvasive **l**obular
IL	**I**nter**l**eukine
INK4	**In**hibitor of CD**K4**
IP3	**I**nositol Tri**p**hosphate
IRS	**I**mmuno**r**eactivity **S**core
KIP	**K**inase **I**nhibitor **P**rotein
LCIS	**L**obular **C**arcinoma **i**n **s**itu
mAB	**m**onoclonal **A**nti**b**ody
MAPK	**M**itogen **A**ctivated **P**rotein **K**inase
MT	**M**elatonin Receptor
NCa	**N**on-**Ca**rcinoma (Tissue)
NOS	**n**ot **o**therwise **s**pecified
OS	**O**verall **S**urvival
PBS	**P**hosphate **B**uffered **S**aline
PI3K	**P**hospho-**I**nositide-**3**-**K**inase
PKA/C	**P**rotein **K**inase **A**/**C**
PLC	**P**hospho**l**ipase **C**
PR	**P**rogesterone **R**eceptor
Rb	**R**etinoblastoma **P**rotein
ROR	**R**etinoid **O**rphan **R**eceptor

RORE	**R**etinoid **O**rphan Receptor **R**esponsive **E**lement
RT	**R**oom **T**emperature
RT-PCR	**R**everse **T**ranscription **P**olymerase **C**hain **R**eaction
RZR	**R**etinoid **Z** **R**eceptor
SERM	**S**elective **E**strogen **R**eceptor **M**odulator
SMA	**S**mooth **M**uscle **A**ctin
SULT	**Sul**fo**t**ransferase
TDLU	**T**erminal **D**uct **L**obular **U**nit
UICC	International Union Against Cancer
VEGF	**V**ascular **E**ndothelial **G**rowth **F**actor
VEGF-R	**V**ascular **E**ndothelial **G**rowth **F**actor **R**eceptor

1 SUMMARY

Breast cancer is the most frequent malignant disease among women worldwide. The term “breast cancer” encompasses many forms, described by histology, differentiation grade and size of the tumor and biomarkers like hormone receptor and HER2 status. These breast cancer types differ in their clinical behavior and aggressiveness, and the assessment of the established biomarkers is sometimes insufficient to accurately predict relapse and survival of the individual patient. Therefore, the search for factors contributing to the development and progression of this disease is an important task.

The malignancy and hence the prognosis of a tumor is significantly influenced by the division rate of the cells of which it is composed. Cell proliferation depends on the frequency of passage through the cell cycle, which is tightly regulated by complexes of cyclins with cyclin-dependent kinases and their inhibitors. Changes in the expression and interplay of cell cycle regulators are considered to be associated with the formation and progression of cancer. Besides steroid hormones like estrogen, recent data suggest that melatonin may also strongly affect cell cycle regulators thereby altering growth and survival of breast cancer cells. So does melatonin suppress the proliferation of breast cancer cells via its binding to the G-protein coupled melatonin receptor, MT1, which is followed by downregulation of estrogen-induced cyclin D1, a protein required for the cell’s transition from G1- to S- phase of the cell cycle. Additionally, other factors like nestin, a marker for neuronal stem cells, may also influence the progression of breast cancer, particularly as in breast tumors, upregulation of nestin expression is found in newly formed blood vessels.

The focus of this thesis was to examine cell cycle regulators, MT1 and nestin with respect to their role as novel biomarkers in the prognosis of relapse and death of breast cancer patients. Another aim was to analyze the expression of melatonin metabolizing enzymes in breast tissue in order to identify patients likely to benefit from the antiestrogenic and antiproliferative properties of melatonin.

The expression of cyclin A, D1 and E, the cyclin-CDK-activating phosphatase CDC25A, the cell cycle inhibitor retinoblastoma protein and the CDK-inhibitors $p16^{INK4a}$,

$p21^{waf1/cip1}$ and $p27^{kip1}$ was immunohistochemically examined in malignant (Ca) and their adjacent non malignant (NCa) tissue specimens from 69 breast cancer patients. Immunohistochemical staining was regarded positive for CDC25A in 36, for cyclin A in 20, for cyclin D1 in 40, for cyclin E in 12, for $p16^{INK4a}$ in 16, for $p21^{waf1/cip1}$ in 25, for $p27^{kip1}$ in 23 and for retinoblastoma protein in 30 of 69 Ca specimens. In NCa, only CDC25A, retinoblastoma protein and $p27^{kip1}$ were regarded to be positive in 41, 21 and 16 of 69 specimens. The expression of cell cycle regulators in Ca or NCa specimens was not related to the time from primary diagnosis to relapse or cancer-associated death. However, expression of CDC25A, cyclin A, cyclin E, $p16^{INK4a}$ and $p27^{kip1}$ in Ca was associated with a shorter overall survival of patients at high risk of relapse than those being at low or intermediate risk.

Breast cancer and adjacent non-malignant tissue specimens from 42 patients were also assessed for MT1 and nestin by double immunofluorescence staining with antibodies against MT1 and nestin. MT1 was detected in luminal and myoepithelial cells of milk ducts in 40 NCa and in tumor cells in 39 Ca specimens. Coexpression of MT1 and nestin was found in myoepithelial cells in 38 NCa and 18 Ca specimens with intact milk ducts. In 22 Ca sections, MT1-nestin coexpression was found in tumor cells infiltrating the stroma and in vascular-like structures. Quantitative analysis of 16 selected paired NCa and Ca samples revealed that the percentage of MT1-expressing cells was higher in 8, and lower in 6 invasive Ca as compared to adjacent NCa sections. MT1-nestin coexpressing cells in more than 10% of tumor cells was found in Ca specimens from patients being in an advanced tumor stage (TII/III) and at a higher risk of relapse.

Immunohistochemical examination of a possible relation between MT1 and cyclin D1 was done in Ca specimens from 33 breast cancer patients, showing staining for MT1 in 31 and cyclin D1 in 27 specimens. Cyclin D1 correlated positively with estrogen receptor expression, whereas no such correlation was found between MT1 expression levels and estrogen receptor status.

Analysis of the mRNA expression of the melatonin-metabolizing enzymes CYP1A1, CYP1A2, SULT1A1 and SULT1A3 in malignant and non-malignant breast tissue specimens from 10 patients by quantitative real-time reverse transcription PCR showed that *CYP1A1* was expressed only in 3 Ca and 1 NCa, while *CYP1A2* mRNA was below the detection limit in all patients. SULT1A1 mRNA was observed only in 2 Ca and 1 NCa specimens from 3 patients, while high SULT1A3 levels were found in all Ca and

NCa samples. Therefore, biotransformation of melatonin to 6-hydroxymelatonin sulfate is not a major pathway in breast cancer.

In summary, cell cycle regulators, MT1 and nestin might have the potential for further refining the assessment of risk of relapse in breast cancer patients.

Moreover, these results indicate that malignant and non-malignant breast tissue differ in their expression of cell cycle regulators, nestin and MT1. The low expression levels of melatonin metabolizing enzymes and high MT1 expression levels in breast cancer tissue strongly argue for a major role of melatonin in the progression of breast cancer.

2 ZUSAMMENFASSUNG

Das Mammakarzinom ist die häufigste Krebsart der Frau weltweit und ist ein Überbegriff für maligne Tumore des Brustdrüsenepithels, die sich makroskopisch, mikroskopisch und molekulargenetisch unterscheiden. Diese Klassifizierung ist von großer Bedeutung da die einzelnen Tumore in ihrer Aggressivität und im Ansprechen auf eine bestimmte Therapie voneinander abweichen. Dennoch reichen die zurzeit etablierten Marker, wie z.B. Östrogenrezeptorstatus oder Tumorhistologie, nicht für die exakte Unterscheidung der zahlreichen Subtypen aus, da der Krankheitsverlauf des Patienten im konkreten Einzelfall anders als erwartet verläuft. Daher ist die Suche nach weiteren Faktoren, die zum Entstehen und Fortschreiten dieser Erkrankung beitragen und damit eine exaktere Vorhersage von Rezidiven, der Überlebenswahrscheinlichkeit sowie Ansprechen auf die Therapie ermöglichen, ein wichtiges Forschungsziel.

Die Aggressivität und Prognose eines Tumors hängt entscheidend von der Teilungsrate seiner Zellen ab. DNA-Verdoppelung und Zellteilung werden im Zellzyklus von Komplexen von Cyclinen mit Cyclin-abhängigen Kinasen gesteuert, die durch die Phosphatase CDC25A aktiviert oder durch CDK-Inhibitoren, wie $p16^{INK4a}$, $p21^{waf1/cip1}$ oder $p27^{kip1}$, gehemmt werden. Die Cycline D und E sind für die fortschreitende Phosphorylierung und damit Inaktivierung des Retinoblastoma Proteins verantwortlich, was zur Freisetzung von E2F Transkriptionsfaktoren und damit zum Eintritt der Zelle in die S-Phase führt. Eine Veränderung der Expression oder Aktivität dieser Zellzyklusregulatoren ist daher mit der Entstehung und dem Wachstum von Tumoren assoziiert. Neue Studien zeigten, dass das Neurohormon Melatonin die östrogen-induzierte Expression von Cyclin D1 und somit die Proliferation von Brustkrebszellen hemmt. Entscheidend für die wachstumshemmende Wirkung von Melatonin in Brustkrebszellen ist dabei die Expression des G-Protein gekoppelten Melatoninrezeptors MT1. Ein Protein, das vor allem in Tumorzellen und Gefäßneubildungen in hochaggressiven, schnell wachsenden Brusttumoren exprimiert ist, ist das Intermediärfilamentprotein Nestin. Nestin wurde ursprünglich in neuronalen Stammzellen identifiziert, in welchen die Aktivierung von MT1 zu erhöhter Proliferation und einer Hemmung der Apoptose führte.

Das Ziel dieser Arbeit war die Untersuchung von Zellzyklusregulatoren, MT1 und Nestin und ihrer Rolle in der Pathogenese von Brustkrebs und als mögliche neue

Biomarker in der Vorhersage von Rezidiven und der damit verbundenen Überlebenswahrscheinlichkeit. Im Hinblick auf einen möglichen adjuvanten Einsatz von Melatonin als Substanz mit antiöstrogener und wachstumshemmender Wirkung in der Brustkrebstherapie war die Untersuchung von melatonin-metabolisierenden Enzymen im Brustkrebsgewebe ein weiteres wichtiges Ziel dieser Arbeit.

Der erste Schritt dieser Arbeit umfasste die immunhistochemische Analyse der Expression von Cyclin A, D1 und E, CDC25A, $p16^{INK4a}$, $p21^{waf1/cip1}$, $p27^{kip1}$ und Retinoblastoma Protein in malignem (Ca) und angrenzendem nicht-malignen (NCa) Gewebe von 69 Brustkrebspatienten mit hohem, mittlerem oder niedrigem Risiko ein Rezidiv zu entwickeln. Von 69 Ca Schnitten wurden 36 als positiv für CDC25A, 20 für Cyclin A, 40 für Cyclin D1, 12 für Cyclin E, 16 für $p16^{INK4a}$, 25 für $p21^{waf1/cip1}$, 23 für $p27^{kip1}$ und 30 für das Retinoblastoma Protein befunden. Im angrenzenden NCa waren hingegen nur 41 Proben für CDC25A, 21 für $p27^{kip1}$ und 16 für das Retinoblastoma Protein immunhistochemisch positiv, während keine Färbung für alle anderen Zellzyklusregulatoren gefunden wurde. Die immunreaktiven Scores der Zellzyklusregulatoren in Ca und NCa Schnitten waren nicht mit einem früheren Rezidiv oder Tod assoziiert. Allerdings zeigten Patienten mit positiver Färbung von CDC25A, Cyclin A, Cyclin E, $p16^{INK4a}$ und $p27^{kip1}$ aus der Hochrisikogruppe ein kürzeres Gesamtüberleben als Patienten mit geringem bis mittlerem Rezidivrisiko.

Ein weiteres Ziel dieser Arbeit war die Untersuchung von Ca und angrenzenden NCa Gewebsproben von 42 Brustkrebspatienten auf eine mögliche Koexpression von MT1 und Nestin mittels Doppelimmunfluoreszenzfärbung. Eine Immunfluoreszenzfärbung von MT1 in luminalen und myoepithelialen Zellen der Milchgänge war in 40 NCa und in Tumorzellen in 39 Ca Proben zu beobachten. 38 NCa und 18 Ca Schnitte zeigten eine Koexpression von MT1 und Nestin in den myoepithelialen Zellen intakter Milchgänge. Eine Koexpression von MT1 und Nestin wurde weiters in infiltrierenden Tumorzellen und kleinen Blutgefäßen in 22 Ca Gewebsproben beobachtet. Die quantitative Analyse von MT1-exprimierenden Zellen in ausgewählten Ca und dazugehörigen NCa Schnitten von 16 Patienten mittels der Bildanalysesoftware TissueQuest® zeigte, dass die Anzahl an MT1-positiven Zellen in 8 Ca höher und in 6 Ca niedriger als im angrenzenden NCa ist. Ein besonders wichtiger Befund dieser Studie war, dass die Anzahl an Tumorzellen (> 10%) mit MT1-Nestin-Koexpression im Stroma mit einem höheren Tumorstadium (TII/III) und einem höheren Rezidivrisiko assoziiert ist.

Eine mögliche Korrelation von MT1 mit Cyclin D1 im Brustkrebsgewebe wurde mittels immunhistochemischer Färbung von 33 Ca analysiert, und zeigte eine Immunfärbung von MT1 in 31 und von Cyclin D1 in 27 Brustkrebsschnitten. Eine Färbung beider Proteine, MT1 und Cyclin D1, wurde in 25 Ca Proben nachgewiesen, jedoch korrelierte der immunreaktive Score von MT1 nicht mit jenem von Cyclin D1.

Die Expression der Melatonin-metabolisierenden Enzyme Cytochrom P450 (CYP) 1A1, 1A2 und der Sulfotransferasen SULT1A1 und SULT1A3 in Ca und NCa von 10 Brustkrebspatienten wurde mittels quantitativer Real-Time Reverse-Transkriptions-Polymerasekettenreaktion untersucht. *CYP1A1*-mRNA war nur in 3 Ca und einem NCa exprimiert, während *CYP1A2*-mRNA in keiner Gewebsprobe enthalten war. *SULT1A1*-mRNA wurde in 2 Ca und einer NCa Probe gefunden, die jedoch nicht mit den Patienten mit *CYP1A1* Expression ident waren. Hingegen zeigten alle Ca und NCa eine Expression von *SULT1A3*-mRNA. Da die Hydroxylierung von Melatonin durch CYP die Voraussetzung für die nachfolgende Sulfatierung durch SULT ist, erlauben diese Ergebnisse die Schlussfolgerung, dass im Brustgewebe keine nennenswerte Metabolisierung und damit Inaktivierung von Melatonin zu 6-Hydroxymelatoninsulfat stattfindet.

Zusammenfassend gibt die Bestimmung von Zellzyklusregulatoren, MT1 und Nestin in Brustkrebsgewebe wichtige Zusatzinformationen über das individuelle Risiko eines Brustkrebspatienten hinsichtlich Metastasierung und der damit verbundenen kürzeren Überlebenszeit. Darüber hinaus zeigen die Ergebnisse dieser Arbeit, dass sich malignes und nicht-malignes Brustgewebe in der Expression von Zellzyklusregulatoren, MT1 und Nestin unterscheiden. Die geringe Expression von Melatonin-metabolisierenden Enzymen bei gleichzeitig hoher MT1-Expression im Brustkrebsgewebe deuten auf eine bedeutende Rolle von Melatonin in der Pathogenese von Brustkrebs.

3 INTRODUCTION

3.1 Breast Cancer

3.1.1 Anatomy and Histology of the Female Breast

A. Anatomy

The female breast is made up of a branching duct system, leading from the nipple to the milk glands. Up to twenty glandular units, termed breast lobes, are arranged in a radial pattern around the nipple (mammary papilla), and are surrounded by a dense, collagenous connective tissue. Lactiferous ducts connect each lobe with the mammary papilla, where they exit the breast via a secretory pore.

Each lobe is composed of several smaller lobules, which are separated from each other by a loose, fibrous fatty connective tissue. The lobules consist of about twenty small glandular structures (acini) which drain through the terminal duct. The entity of acini and terminal duct is called the “terminal duct-lobular unit” (TDLU). During pregnancy, the alveolar ductules of the TDLU differentiate into the milk-producing acini (Figure 1) (Junqueira et al 1996).

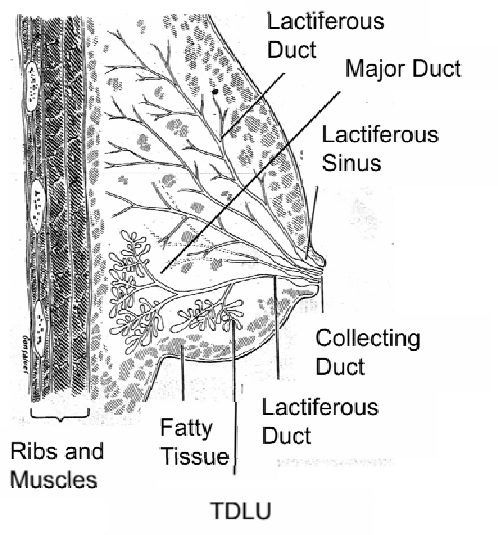

Figure 1. Anatomy of the Female Breast. Branching system of ducts leading down from the nipple to the terminal duct lobular units (TDLU), which is composed of small milk glands. (Junqueira et al 1996)

B. Histology

The mammary gland is composed of glandular tissue and its surrounding connective tissue.

In the glandular tissue, the ducts are made up of a single layer of epithelial cells lining the lumen (luminal cells), which is surrounded by a continuous layer of myoepithelial cells. The latter separates the luminal cells from the basement (basal) membrane and the stroma. By contrast, in the alveoli, some luminal epithelial cells are in direct contact with the basal membrane (Polyak and Hu 2005) (Figure 2).

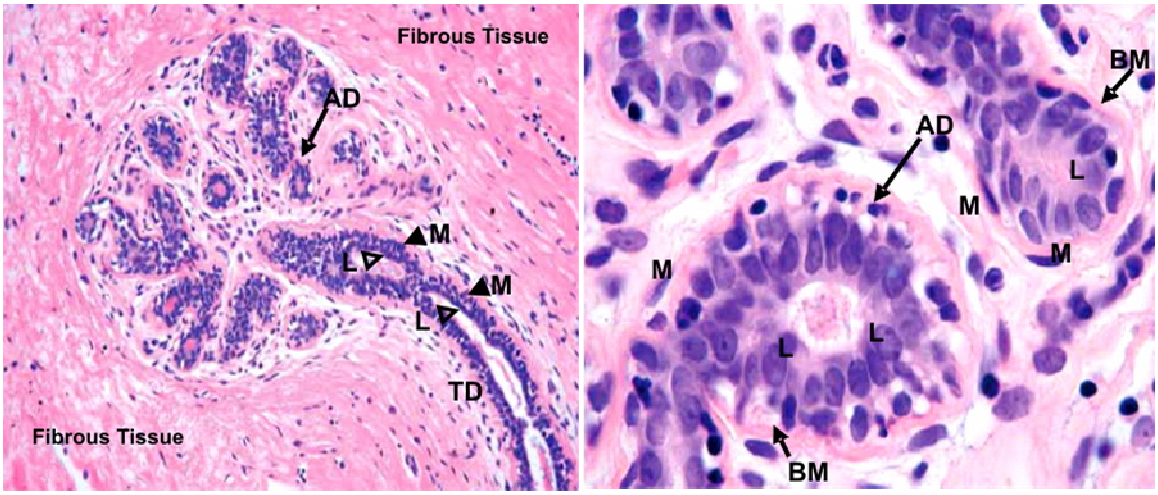

Figure 2. Sections from healthy human breast tissue, showing a terminal milk duct (TD), branching into several small, milk-producing, alveolar ductules (AD) (left, 25x magnification). The right picture shows the alveolar ductules at higher (250x) magnification. Both, terminal duct and alveolar ductuli are composed of an inner layer of secretory luminal cells (L) and an outer layer of myoepithelial cells (M) which adjoins to the basement membrane (BM). The basement membrane separates the ducts and ductules from the surrounding fatty fibrous connective tissue. Blue hematoxylin-staining denotes the cell nuclei, while the cytoplasm is stained red by eosin. (Moinfar 2007)

The connective tissue, which surrounds the glandular tissue, is composed of blood and lymphatic vessels, nerves, adipose and fibrous tissue, and therefore multiple cell types, such as endothelial cells, smooth muscle cells, fibroblasts, blood cells, nerve cells and adipocytes are found in the breast stroma (Guinebretiere et al. 2005).

3.1.2 Breast Cancer and its Histological Forms

Breast cancer is the most common form of cancer among women, and makes up one third of all new cancer cases in Europe (reviewed by Karim-Kos et al 2008). Although the mortality rates have declined in the past two decades due to earlier detection and better treatment options, the morbidity is still increasing (Hilakivi-Clarke et al. 2004).

The most common malignancies of the breast are adenocarcinomas arising from the epithelium of either the lobules or ducts. This can be explained by the fact that the mammary luminal epithelium is hormone-responsive due to its expression of estrogen- and progesterone-receptors, and underlies continuous changes within each menstrual cycle (reviewed by Clarke 2003).

Around 50% of all breast carcinomas are located in the upper outer quadrant of the breast (Lee 2005).

Histologically, various steps can be identified during the formation and progression of breast carcinomas. The increased proliferation of luminal epithelial cells results in ductal hyperplasia, which is still a benign condition. The cells may then transform to premalignant atypical cells with nuclei of varying shape and size, giving rise to the formation of a non-invasive, in situ, carcinoma being still confined to the milk ducts and lobules (reviewed by Purcell and Norris 1998). When the cells break through the basement membrane and the primary tumor spreads into the surrounding stroma, the tumor has become an “invasive carcinoma”. Now, the tumor cells may also invade lymphatics and blood vessels, giving rise to metastases in locoregional lymph nodes, but also in distant organs (reviewed by Beckmann et al 1997).

Due to their cellular morphology and tumor growth pattern, many distinct histological forms of breast cancer exist. In the following, the histological features of all breast carcinomas relevant for this thesis will be described.

Non-invasive breast cancers

A. Ductal carcinoma in Situ (DCIS)

The intraductal carcinoma is found in around 15-30% of carcinomas, and is four times more common than the lobular carcinoma in situ. DCIS results from proliferation of epithelial cells of the TDLU. Due to its high proliferation, the tumor in the TDLU extends, so that the terminal ducts resemble large ducts (Figure 3). Five morphological types of DCIS can be distinguished: solid, comedo, cribriform, papillary and micropapillary DCIS. These morphologic types differ greatly in their risk to become invasive, with comedocarcinoma having the highest and pure cribriform or micropapillary carcinomas having the lowest risk (Tavassoli 1992).

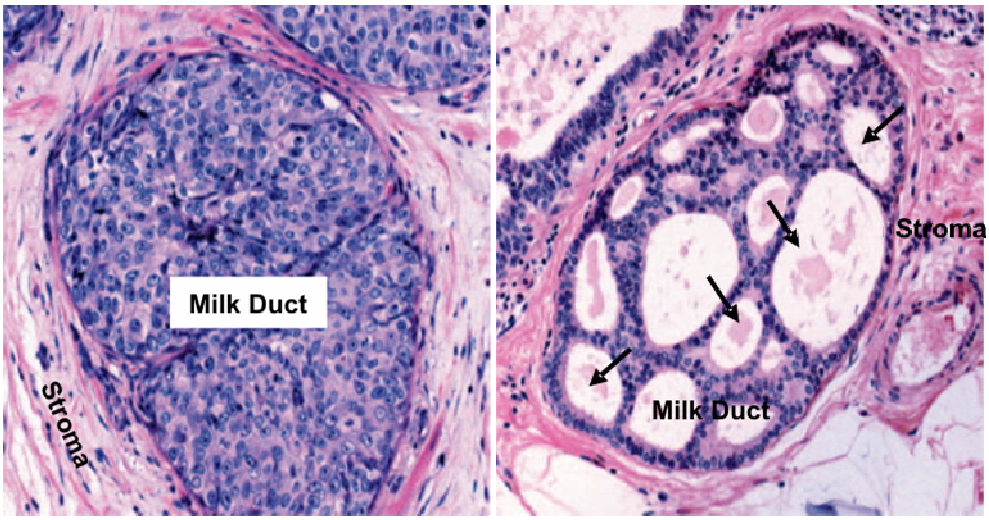

Figure 3. Sections from breast carcinomas which are still confined to the milk ducts, without breaking in the surrounding stroma (ductal carcinoma in situ, DCIS), 20x magnification. Hematoxylin-blue stained tumor cell nuclei fill the milk ducts in a solid (left) and a cribriform (right) pattern of DCIS. In the cribriform DCIS, the tumor cells form fenestrations (arrows). Cytoplasma is stained red with eosin.
(Sun et al. 2009)

B. Lobular Carcinoma in Situ (LCIS)

Like DCIS, non-invasive lobular carcinoma originates in the TDLU. LCIS does not form a palpable mass and is therefore only randomly detected in patients biopsied for other reasons. This in situ carcinoma is very often multifocal and simultaneously found in both breasts (bilateral). If LCIS is left untreated, about one third of the lesions will become invasive within twenty years. Microscopically, LCIS is characterized by small uniform cells with sparse cytoplasm that fill the TDLU. Very few mitoses are seen (Tavassoli 1992).

Invasive breast cancers

Invasive carcinomas may be classified according to the relationship of tumor mass to the amount of connective tissue. Accordingly, medullary carcinomas contain a high tumor proportion, and scirrhous carcinomas a high amount of connective tissue. The carcinoma simplex contains equal proportions of tumor and connective tissue.

Carcinomas with characteristic histological features are called invasive carcinomas of special type, and examples are the lobular, medullary, mucoid, tubular and apocrine invasive carcinoma. In the case of no special histological features, the carcinoma is designated *“carcinoma not otherwise specified”, NOS* (Moinfar 2007).

C. Invasive Ductal Carcinoma, NOS

About 80% of breast carcinomas are of the invasive ductal (id) NOS type. Histologically, the tumor cells are larger than normal epithelial cells, and cluster to tubules or sheets (Figure 4). Mitoses, necrosis and calcification are often observed (Tavassoli 1992).

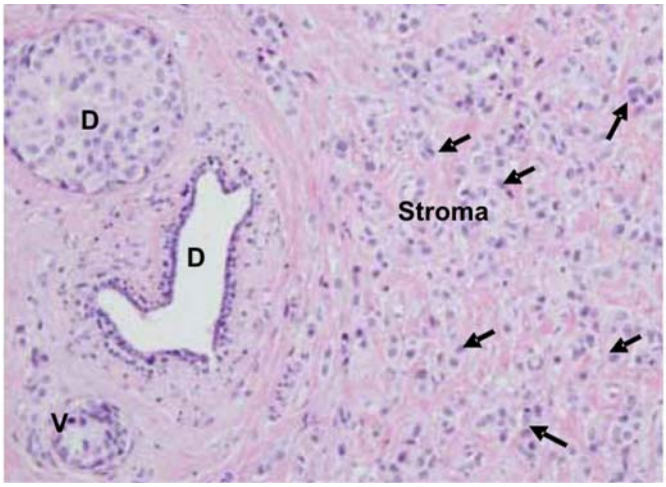

Figure 4. Section from an invasive breast carcinoma originating from the large milk ducts, 100x magnification. D denotes a milk duct which is filled with blue-stained tumor cells (at the top, left) or which still shows a non-malignant appearance (center). The tumor cells (arrows) have spread beyond the basement membrane of the ducts into the surrounding stroma. V, blood vessel. Blue, hematoxylin-stained cell nuclei; red, eosin-stained cytoplasma and stroma. (Song et al. 2006).

D. Invasive Lobular Carcinoma

Invasive lobular (il) carcinoma accounts for about 10% of all invasive breast carcinomas and is the second most common type of invasive carcinoma. Infiltrating lobular tumors are often impalpable. In up to one third of cases, concurrent invasive carcinoma in the other breast might be possible. Microscopically, invasive lobular carcinomas consist of small uniform tumor cells which infiltrate as individual rows (Indian file) or show a concentric arrangement (Figure 5) (Tavassoli 1992).

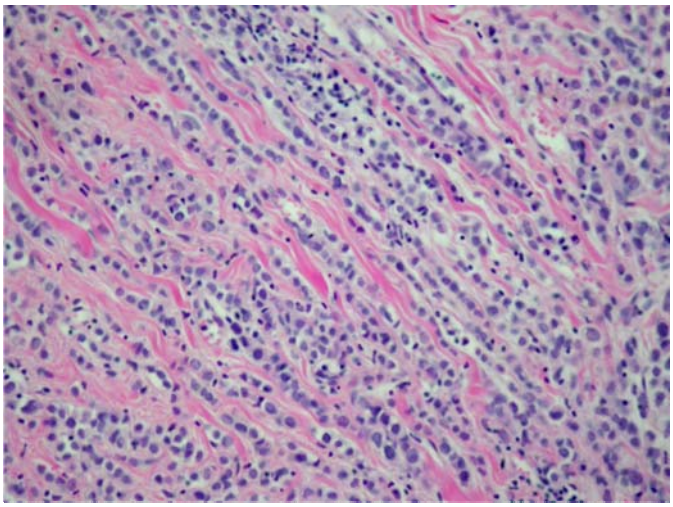

Figure 5. Section from an invasive breast carcinoma originating in the lobules, 200x magnification. This invasive lobular carcinoma is characterized by nearly uniform small tumor cells infiltrating the stroma in single rows ("Indian files"). Cell nuclei are stained blue with hematoxylin. Cytoplasma and stromal components are stained red with eosin. (Franceschini et al. 2006).

E. Medullary Carcinoma

Around 5-7 % of all breast carcinomas are medullary carcinomas. Histologically, medullary carcinomas are characterized by large solid tumor cell conglomerates which are infiltrated by inflammatory cells. The tumor cells lack defined cell borders (syncytial pattern), and show large nuclei of variable form and shape with pronounced nucleoli. Mitotic activity is high. Hardly any glandular or fatty tissue is found within the invasive tumor (Figure 6) (Wargotz and Silverberg 1988).

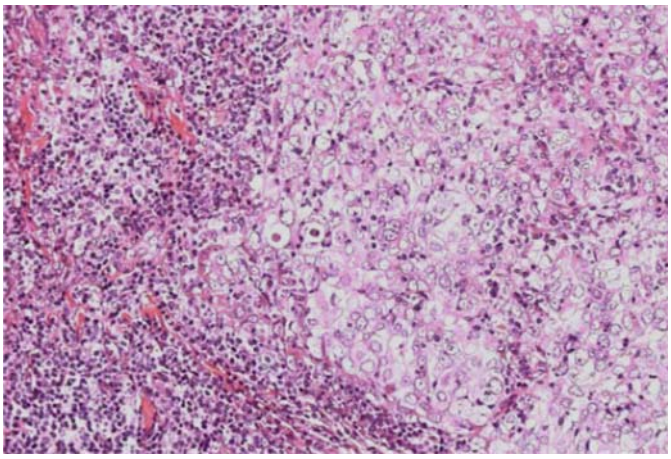

Figure 6. Section from an invasive medullary breast carcinoma, 100x magnification. No milk ducts are seen any more. Large tumor cells of varying size and shape are spread throughout the stroma. The tumor cells have pronounced nucleoli. Mitoses are commonly observed. The stroma is infiltrated with small, more intensely stained, lymphoid cells (left part of the picture). The blue color indicates hematoxylin-staining of cell nuclei, whereas cytoplasma and stromal components are stained red with eosin. (Milde et al. 2006).

F. Mucinous (Colloid) Carcinoma

This small subset of breast carcinomas (~ 3%) mainly occurs in elderly women of > 75 years of age. Carcinomas of the colloid type are gelatinous and soft in their consistency, and are characterized by tumor cell clusters and single tumor cells floating in pools of extracellular mucin (Figure 7) (Tavassoli 1992).

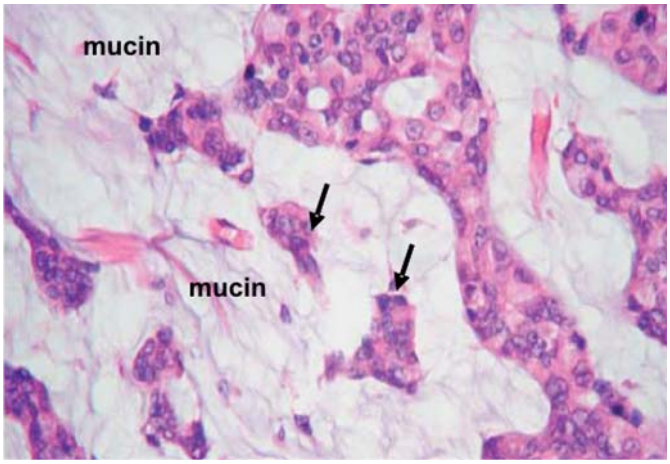

Figure 7. Section from an invasive mucinous breast carcinoma, 100x magnification. No ductal structures are any longer recognizable. Clusters of tumor cells (arrows) are floating in pools of extracellular mucin (unstained). Blue, hematoxylin-stained tumor cell nuclei; red, eosin-stained stromal components. (Moinfar 2007)

G. Tubular Carcinoma

Tubular carcinomas account for about 2% of all invasive breast cancers, and represent a very well differentiated form of ductal carcinoma. Although being often impalpable, these tumors can be usually well detected by mammography. The term “tubular” refers to the arrangement of cells into small glands and tubules of variable shapes, which are randomly distributed in a fibroblastic stroma. The often angulated tubules display open lumens and are lined by a single layer of normally appearing epithelial cells with rare mitotic activity (Figure 8) (Tavassoli 1992).

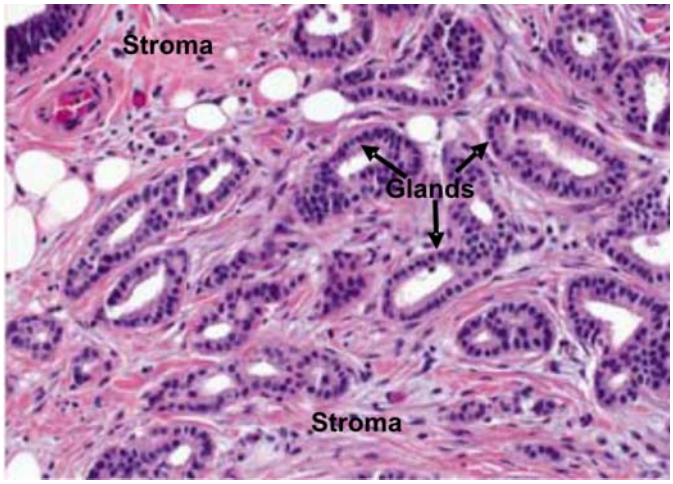

Figure 8. Section from an invasive tubular breast carcinoma, 200x magnification. The glands are made up by a single layer of carcinoma cells; myoepithelial cells have disappeared. The glands infiltrate into the surrounding stroma. Blue, hematoxylin-stained cell nuclei; red, eosin-stained stroma. (Esposito et al. 2007).

H. Apocrine Carcinoma

Apocrine carcinoma is observed in about 0.5% of all breast carcinomas (Celis et al. 2006). This invasive carcinoma is composed of large cells with abundant eosinophilic granular cytoplasm surrounding large, hyperchromatic nuclei with pronounced nucleoli. Some carcinoma cells contain lipid droplets thereby simulating sebaceous cells (Figure 9) (Moinfar 2007).

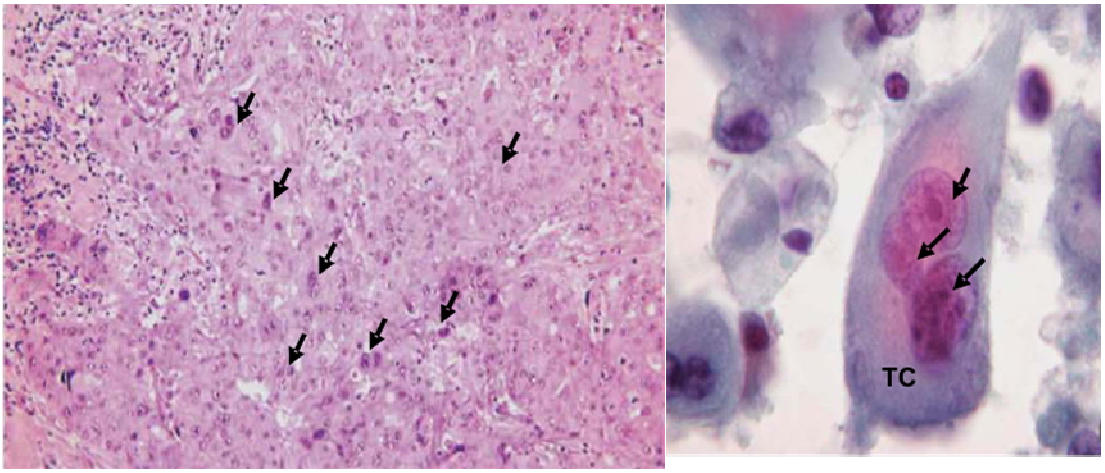

Figure 9. Sections from an invasive apocrine breast carcinoma. The left picture shows large heteromorphic tumor cells (arrows) invading the stroma, at 100x magnification. These cells are intensely stained with eosin, a red dye marking the alkaline components of cytoplasma. The tumor cell nuclei are only weakly stained with hematoxylin (blue). In the upper left part of the picture, the small dark-blue stained cells indicate an infiltration with inflammatory cells. The right picture shows a single tumor cell (TC) with abundant eosinophilic cytoplasm and three nuclei (arrows) of varying size and shape at 2500x magnification. Also, the nucleoli are visible. (Moinfar 2007)

3.2 Prognostic and Predictive Parameters in Breast Cancer

According to the National Health Institute, a biomarker is “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Biomarkers Definitions Working Group 2001). Biomarkers are required for the diagnosis of breast cancer. By their assessment, breast cancer patients should be recognized as early as possible. Moreover, biomarkers should describe the clinical behavior of the tumor in order to identify patients at high risk of relapse and in order to select the optimal therapy. Biomarkers are therefore classified into “prognostic” and “predictive” factors [Oldenhuis et al 2008]. Prognostic parameters are determined in patients prior to chemotherapy and should give information on the patient’s particular course of disease, the risk of relapse and the probability of survival. By contrast, predictive factors should estimate whether a patient would benefit from a particular therapy or not [Oldenhuis et al 2008].

Below, biomarkers already established in routine are briefly described, followed by novel strategies.

3.2.1 Prognostic and Predictive Markers in Breast Cancer Routine Diagnostics

Traditional prognosticators include factors which describe characteristics of the tumor (histology, size, differentiation grade) and the patient (age, menopausal status), and molecular markers, like the receptors for estrogen and progesterone and the human epidermal growth factor receptor 2.

A. Histology

The histological assessment of tumor specimens can contribute to predict the further course of disease, since the diverse histological breast cancer subtypes show a different clinical behavior. Patients with infiltrating ductal carcinomas of no special type have been shown to have the worst prognosis, followed by lobular and medullary carcinomas (Ellis et al. 1992). Pure mucinous carcinomas grow relatively slowly, so that 10 years after primary diagnosis, more than 90% of patients are still alive (Feiner and Moezzi 1999). Also, tubular carcinomas show a favorable prognosis (Ellis et al. 1992). Studies assessing the prognosis of patients suffering from apocrine carcinoma are contradictory (Celis et al. 2006). Apocrine carcinomas are often undifferentiated, high-grade tumors, a feature per se known to have a poor prognosis. However, the survival of patients with apocrine carcinoma does not seem to differ from other, non-apocrine, high grade breast carcinomas (Moinfar 2007).

B. Grade

The histological grade is an indicator for the degree of differentiation of a tumor. Well-differentiated, low-grade, tumors still resemble the tissue of origin while there is no such similarity observed for poorly differentiated, high-grade, tumors. There is a close relationship between the degree of differentiation and the aggressiveness of a neoplasm, with poorly differentiated tumors showing a higher proliferation rate and propensity for invasiveness than well differentiated ones. Therefore, the assessment of the tumor grade provides important prognostic information. Several grading systems are currently in use for breast cancer, of which the most common are the Scarff-Bloom-Richardson (SBR) system and the more modern Elston-Ellis modification of the SBR grading system (Nottingham grading system) (Bloom and Richardson 1957; Elston and Ellis 1991). They combine the extent of variation in size and shape of the tumor cells (nuclear pleomorphism), the presence of milk ducti (tubule formation) and the frequency of mitoses. The assessment of the tumor differentiation grade is shown in Table 1.

Features	Criteria	Score
Tubule formation	> 75 %	1
	10 – 75 %	2
	< 10 %	3
Mitotic Count	< 10 mitoses/10 HPF	1
	10 – 19 mitoses/10 HPF	2
	≥ 20 mitoses/10 HPF	3
Nuclear Pleomorphism	Small regular uniform cells	1
	Moderate nuclear size and variation	2
	Marked nuclear variation	3
Sum of Scores		**3 - 9**
	Grade	**Terminology**
3 – 5	G1 (”low grade”)	Well differentiated
6,7	G2 (“intermediate grade”)	Moderately differentiated
8,9	G3 (“high grade”)	Poorly differentiated

Table 1. Evaluation of the tumor differentiation grade according to Bloom and Richardson (Bloom and Richardson 1957). The breast cancer sections are viewed under a microscope at maximum magnification (HPF, high power field). For assessment of the grade, the following parameters are taken into consideration: the extent of glandular formation (tubule formation), the number of dividing cells (mitotic count) and size and shape of the tumor cell nuclei (nuclear polymorphism). To each of these parameters, a score from 1 to 3 is assigned and the scores are summarized to give the histological grade.

C. Tumor Stage

The cancer stage provides information on the extent of spread of the tumor and is regarded the most important predictor of survival (Marrazzoa et al. 2007). The most common staging system for breast cancer, used by the International Union against Cancer (UICC), the American Joint Committee on Cancer (AJCC) and the International Federation of Gynecology and Obstetrics (FIGO), is the TNM staging, which is an abbreviation for **t**umor, **n**ode and **m**etastases. Tumor, T, refers to the size of the primary tumor and is given a number from 0 (≤ 2 cm) to 4 (> 5 cm). N represents the number and localization of regional lymph nodes with metastases and is ranked from 0 (no metastases in nodes) to 4 (Sobin and Wittekind 2002).

Remarkably, patients showing invasion of tumor cells into locoregional lymph nodes have a significantly poorer prognosis than those without nodal metastases (Fitzgibbons et al. 2000). Metastasis into distant organs is represented by the letter M, and is 0 if the tumor has not spread to distant organs or 1 if metastases are present (Sobin and Wittekind 2002). Additionally, very often, invasion into lymphatic (L) and blood vessels (V) is also evaluated and indicated (Sobin and Wittekind 2002).

These factors (Tumor, Nodes, Metastases, Lymphatics and Venes) are then summarized to give the stage, which is expressed in Roman numerals. Stage I cancers are localized to one part of the body, while stage II and III cancers are locally advanced. Stage IV cancers have spread to other, more distant, organs.

D. Estrogen and Progesterone Receptors (ER and PR)

As estrogen and progesterone play an important role in breast physiology, they are also involved in its pathophysiology. Their effects are predominantly mediated via binding to their nuclear receptors ER and PR, which, upon activation, form dimers and function as transcription factors to regulate the expression of specific genes involved in cell proliferation (Figure 10). Remarkably, one ER target gene encodes for PR, and estrogen was shown to upregulate PR expression (Clarke 2004). The estrogen effects are mediated via two ER-subtypes, namely ER-α and ER-β, which are encoded by two different genes, giving rise to proteins which are very similar in their DNA binding domain but differ in about 42% in their ligand binding domain (Ruff et al. 2000). High levels of ER- β have been found in resting breast epithelium, while in proliferating breast tissue these levels are only low (Lofgren et al. 2006). Moreover, ER-β has been shown to counteract the proliferative effects of ER-α (Paruthiyil et al. 2004). In analogy to ER, two subtypes of PR, PR-A and PR-B, encoded by a single gene, have been identified. It has been shown that PR-B activation results in enhanced cell proliferation which can be antagonized by PR-A (Conneely et al. 2000).

Selective estrogen receptor modulators (SERM) have a different affinity for ER-α and ER-β, and are effectively applied as adjuvant drugs in breast cancer chemotherapy. To predict the response to this endocrine treatment, the expression of both, ER and PR, is routinely determined by immunohistochemical (IHC) staining of breast cancer tissue sections in which the localization and distribution of ER and PR

is detected via binding of specific antibodies (Ravdin et al. 1992; Cui et al. 2005; Hayashi and Yamaguchi 2005). In addition to their role in prediction of therapy, the presence of hormonal receptors is also a favorable prognostic factor for overall survival. In this context, it has been shown that patients with ER-positive- but PR-negative- tumors have a worse overall survival than those with carcinomas positive for both, ER and PR (Balleine et al. 1999).

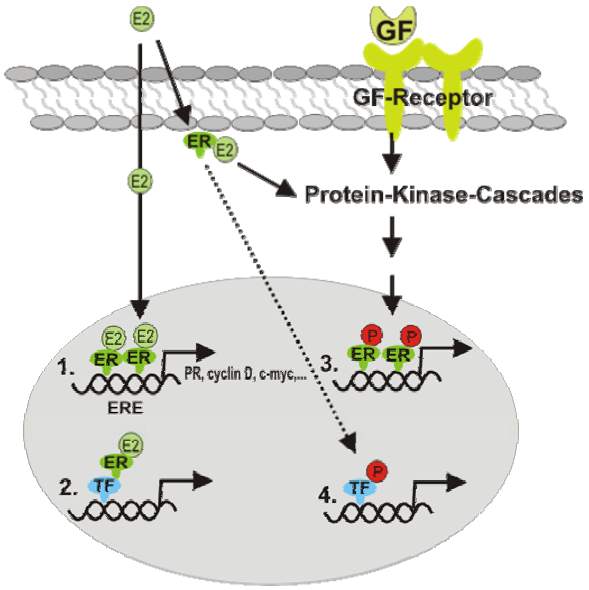

Figure 10. Estrogen signaling pathways. Estradiol (E2) passes the cell membrane and binds to its estrogen receptor (ER) in the nucleus. This is followed by formation of ER-homodimers, which function as transcription factors by binding to a specific target gene region, the estrogen responsive element (ERE), and starting with the transcription of genes like cyclin D1, progesterone receptor (PR), etc (1). Apart from its role as transcription factor, the E2-ER complex may also recruit other transcription factors (TF), thereby transactivating specific genes (2). In addition to E2, also growth factors (GF) may indirectly activate nuclear ER. GF bind to their membrane GF-receptors and activate protein-kinase cascades, leading to phosphorylation (P) and activation of nuclear ER (3). E2 may also bind to an estrogen binding site located in the cell membrane, leading to altered functions of cytoplasmic proteins followed by phosphorylation and activation of transcription factors (4). (Bjornstrom and Sjoberg 2005).

E. Patient Age and Menopausal Status

Other important parameters for the prognosis and selection of therapy are the age and menopausal status of the breast cancer patient. Several studies have shown that younger age (< 35) is associated with more aggressive tumors, a higher risk of relapse and a shorter overall survival (Schmidt et al. 2009). In particular, the menopausal stage influences the treatment strategy for hormone therapy. Since estrogen is primarily produced in the ovaries in premenopausal women, they receive

SERM together with ovarian suppressors. In postmenopausal patients, estrogens are mainly synthesized from androgens by the enzyme aromatase in the fatty tissue, and these patients are therefore treated with a combination of SERM with aromatase-inhibitors.

F. Human Epidermal Growth Factor Receptor 2 (HER2)

HER2, also known as ErbB2 or Neu, is a membrane-spanning receptor tyrosine kinase, and is overexpressed in 20-30% of breast cancers (Harari and Yarden 2000). Upon stimulation, HER2 is recruited as co-receptor for other ErbB-receptors of the same family, and transduces its signals by subsequent autophosphorylation, which activates survival and mitogenic pathways such as ras-raf-MEK-Extracellular Signal Regulated Kinase (ERK) or phosphatidylinositol 3-kinase (PI3K)-Akt (Albanell et al. 2003) (Figure 11). Overexpression of HER2 results either from gene amplification or mRNA overexpression and is associated with a higher probability of relapse and resistance to chemotherapy (Harari and Yarden 2000). Because of its prognostic role as well as its ability to predict response to antibodies (trastuzumab) or drugs (lapatinib, pazopanib) targeting the HER2 protein (Nanda 2007), breast tumors are routinely checked for overexpression or gene amplification of HER2. Three types of assays are currently used, namely IHC to measure the HER2 protein and fluorescence or chromogenic *in situ* hybridization (FISH or CISH) to detect HER2 gene amplification. The *in situ* hybridization method applies dye-linked probes which specifically bind to the DNA sequences encoding for HER2, followed by detection via fluorescence or light microscopy. In routine, the HER2 status is mostly determined with a two-step method, with FISH or CISH used to confirm only moderate staining of IHC. All other cases are either negative (no or weak IHC staining) or positive (strong IHC staining) for HER2 (Cuadros and Villegas 2009).

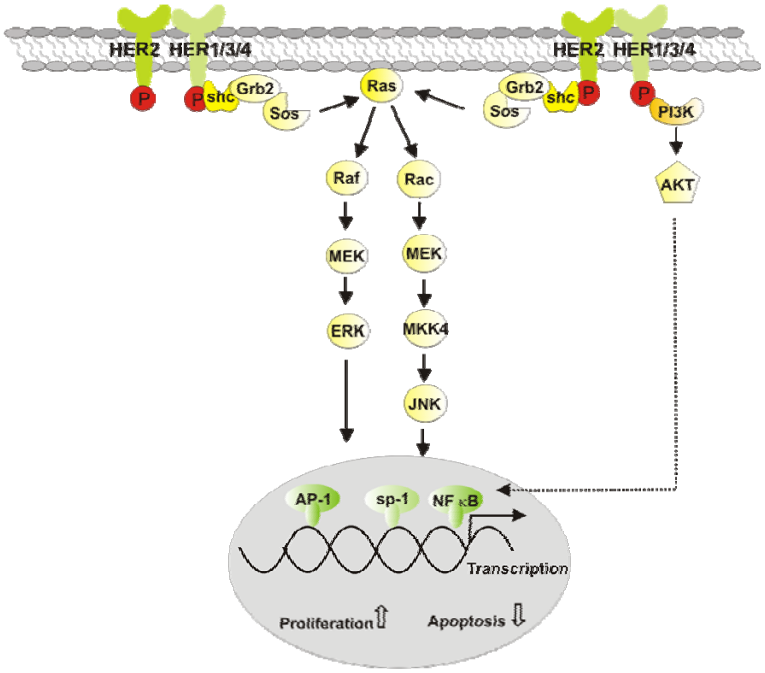

Figure 11. HER2 signaling pathways. Upon binding of epidermal growth factor to the human epidermal growth factor receptors (HER) 1, 3 or 4, HER2 is recruited as co-receptor, followed by autophosphorylation (P) of the receptor dimer. The adaptor molecules src-homology-and-collagen-containing-protein (shc) and growth-factor-receptor-bound (Grb) bind to the phosphorylated tyrosine residues of the receptor dimer and activate the protein son-of-sevenless (Sos), forming all together a multiprotein scaffold. This multiprotein scaffold alters the affinity of Ras for guanosine diphosphate which is exchanged by guanosine triphosphate, thereby activating Ras. Ras turns on mitogen-activated protein kinase kinases (MEK) to phosphorylate the extracellular-signal –regulated-kinase (ERK) or c-Jun-N-terminal-kinase (JNK), acting on the transcription factors activator-protein-1 (AP-1), sp-1 and nuclear-factor-kappa-B (NFκB) to start with the transcription of genes involved in cell proliferation and survival. Activated HER can also stimulate the phosphatidylinositol-3-kinase (PI3K), which phosphorylates inositol-containing lipids of the cell membrane. This recruits the protein-kinase-B (Akt) which phosphorylates numerous substrates, leading to activation of NFκB and transcription of antiapoptotic proteins. (Weinberg 2007).

The prognosis of patients with breast cancer is determined by a combination of the above described factors. In the St. Gallen Consensus Conference 2005, a panel of international breast cancer experts elaborated a novel stratification system with regard to the patient's risk of local or distant recurrence. Based on data on patient age, tumor size, presence of tumor cells in local lymph nodes, tumor differentiation grade, HER2 status, and peritumoral vascular invasion, the patients are assigned to be at low, intermediate or high risk of local or distant recurrence (Table 2) (Goldhirsch et al. 2005).

Low risk	***Node negative AND all of the following features:*** Pathologic tumor size ≤2cm, AND Grade 1, AND Absence of peritumoral vascular invasion, AND HER2/neu gene neither overexpressed nor amplified, AND Age ≥35 years
Intermediate risk	***Node negative AND at least one of the following features:*** Pathologic tumor size >2cm, OR Grade 2-3, OR Presence of peritumoral vascular invasion, OR HER2/neu gene overexpressed or amplified, OR Age <35 years ***Node positive (1-3 nodes involved) AND*** ***HER2/neu gene neither overexpressed nor amplified***
High risk	***Node positive (1-3 nodes involved) AND*** ***HER2/neu gene overexpressed or amplified*** ***Node positive (4 or more involved nodes)***

Table 2. Prognosis of the likelihood to relapse for a patient with operated breast cancer. According to the St. Gallen expert meeting, the patients are assigned to risk categories depending on their age, tumor size and grade, spread of tumor cells to local lymph nodes or blood vessels and HER2 status (Goldhirsch et al. 2005).

3.2.2 Novel Strategies

The exclusive assessment of already established prognostic factors sometimes fails to predict the course of disease. Moreover, the exact mechanisms involved in the initiation and progression of breast cancer have not yet been fully identified. Therefore, one of the challenges in tumor research is finding new biomarkers which should refine the prognosis of a patient's particular course of disease, estimate his risk for recurrence and his chance of survival. Also, new biomarkers could play a role as target for novel drugs and could identify patient subgroups likely to benefit from a certain therapy.

With respect to this thesis, two novel strategies are mentioned now, although their prognostic value and feasibility of assessment in routine diagnostics have to be further evaluated.

A. Angiogenesis

Tumor growth requires the delivery of oxygen and nutrients via blood vessels. Therefore, the formation of new blood vessels, termed angiogenesis, goes along with the growth of the tumor. Moreover, angiogenesis is associated with the spread of the tumor to distant organs, because the increase in the number of blood vessels in the tumor surrounding increases the chances for tumor cells to enter circulation.

Hypoxic conditions in the tumor tissue prompt the tumor to secrete growth factors, like the vascular endothelial growth factor (VEGF), which stimulate the proliferation and motility of endothelial cells in the surrounding capillary network (Sessa et al. 2008; Phng and Gerhardt 2009). However, not all endothelial cells required for the new vessel originate from neighboring vessels, but also from the recruitment of circulating bone-marrow derived endothelial precursor cells (Sullivan et al. 2009).

The angiogenic status of a tumor critically influences the patient's survival and can be assessed by different approaches. The microvessel density has been shown to be a good estimate for the angiogenic status of the tumor and is assessed by IHC staining with antibodies directed against endothelial antigens (CD31, CD34, factor VIII related antigen) followed by manual or computer-based counting of the stained structures in relation to the tumor area under the microscope (Sullivan et al. 2009). According to recent studies, the microvessel density is associated with larger tumors,

presence of lymph node metastases, negative hormone receptor status and shorter survival of breast carcinoma patients (Mohammed et al. 2009; Sullivan et al. 2009). Besides their prognostic role, parameters describing neovascularization might also predict the response to antiangiogenic compounds (Sessa et al. 2008).

B. Molecular Profiling

The gene profile microarray technique permits the concomitant analysis of multiple genes thereby improving the molecular characterization and also the understanding of signaling pathways in breast cancer (Ramaswamy and Golub 2002). This technique is based on the hybridization of prefabricated nucleic acid polymers, immobilized on a solid surface, with complementary gene sequences in the samples to be analyzed. In the two-color microarrays, the cDNAs of the sample and a reference sample are labeled with two different fluorescence dyes and then hybridized with the probes containing the respective sequence. Depending on the ratio of gene expression between sample and reference sample for a given gene, either none, one or both cDNAs are bound to their complementary probes, giving rise to different colors of varying intensity in the respective microarray spot. The fluorescence of these spots is measured, and the thus obtained data representing expression levels of thousands of genes are analyzed with a hierarchical clustering algorithm. Therefore, samples showing similarities in their overall gene expression are grouped together (Perou et al. 1999).

According to their similar gene expression pattern, Perou et al defined four molecularly and clinically different breast cancer subtypes: luminal -, HER2-, normal breast- and basal-like (Perou et al. 2000). The study was performed on 36 infiltrating ductal and 2 lobular carcinomas, 1 ductal carcinoma in situ, 1 fibroadenoma (a benign breast lesion) and 3 normal breast samples. The gene expression patterns of these samples were compared to that of 11 cultured cell lines which served as reference, including cells derived from breast carcinoma, liposarcoma, T-cell leukemia, multiple myeloma and promyeloic leukemia, among others. Further examination of an extended data set showed that the luminal subgroup could be subclassified into luminal A- and luminal B-like cancers (Sorlie et al. 2001). The luminal subtypes make up 67% of all breast tumors, and are characterized by their expression patterns reminiscent of the luminal epithelial cells in the breast since they show high expression of ER-α and the cytokeratins (CK) 8 and 18, but a low

expression of HER2 (Perou et al. 2000). CK are intermediate filaments differentially expressed in epithelial cells dependent on the cell type and its differentiation status [reviewed by Quinlan et al 1985]. Compared to luminal A tumors, the luminal B subtype shows a higher expression of genes related to cell proliferation, like CCNB1, MKI67 and MYBL2 (Cheang et al. 2009). No expression of hormone receptors is seen in tumors of the HER2 subtype, which are characterized by overexpression of HER2 and frequent mutations (up to 80%) of the tumor suppressor p53. Moreover, high grade tumors show more likely features of the HER2- than the luminal subtype (Sorlie et al. 2001). The fibroadenoma together with the normal breast tissue specimens were clustered to the normal breast subtype. Tumors assigned to the normal breast subtype showed high expression of genes characteristic for adipose cells, like fatty-acid binding protein 4 or peroxisome proliferators-activated receptor gamma (PPAR-γ) (Sorlie et al. 2001). Basal-like tumors represented around 15% of the specimens analyzed, and show an expression pattern similar to myoepithelial cells of the normal breast, i.e. strong expression of the basal CK 5, 6 and 17, and lack of hormone receptor- and HER2-expression (Sotiriou et al. 2003). Interestingly, carriers of BRCA-1 mutations often develop carcinomas of the basal type (Turner et al. 2004).

Importantly, this classification of tumors according to their gene expression profile, has been shown to be of clinical relevance as the five cancer subtypes, namely luminal A- and B-, HER2-, normal breast- and basal-like, significantly differ in their prognosis and therapy response, ranging from the best-prognosis luminal A- to the worst-prognosis basal-like tumors, which show a high propensity for brain metastases (Sorlie et al. 2001, Fadare and Tavassoli 2008).

Since gene expression microarrays are expensive, a more convenient approach, namely IHC microarrays, has been developed. IHC microarrays are composed of paraffin-embedded tissue cores of about 0.6 mm in diameter spaced in an array pattern in a recipient paraffin block. From this block, sections are cut, mounted on adhesive slides, and each slide is subjected to immunohistochemical staining. Therefore, this technique allows the simultaneous analysis of many proteins (up to 500, depending on the thickness of the sections) in a huge number of breast tumor samples, and according to the pattern of protein expression, in analogy to gene expression profiling, biologically distinct classes of breast cancer were defined (Abd El-Rehim et al. 2005). However, to date there is no international consensus on the

IHC definition of basal-like breast carcinomas since different studies have assessed different markers (Fadare and Tavassoli 2008). In most tissue microarray studies, tumors are classified as basal-like when being triple-negative for ER-, PR- and HER2, while showing immunostaining for the basal markers CK-5 and -6 and the epidermal growth factor receptor (EGFR) (Fadare and Tavassoli 2008). Histologically, basal like breast carcinomas mostly belong to invasive ductal or medullary tumors showing a high mitotic activity and therefore a high grade.

To summarize, molecular profiling by gene expression and IHC microarray analysis can provide important information about key molecular events in breast cancer and may give a molecular explanation for the different histological phenotypes and their impact on the course of disease. Together with established markers, like ER, PR, HER2, tumor size, grade and lymph node stage, these techniques may thus permit a more accurate prediction of survival and also of therapy response (Hu et al. 2006).

3.3 Cell Cycle Regulators – Impact on Proliferation

Carcinogenesis and tumor growth are the result of uncontrolled cell division. Therefore, factors controlling the replication and division of a cell could be of relevance as novel biomarkers for breast cancer.

The interval between two cell divisions, the cell cycle, is composed of four phases, the G_1, S, G_2 and M-phase. In the DNA synthesis (S)-phase, the cell duplicates its genetic information, and then divides in two in the mitosis (M)-phase. Two gap phases, G_1 (between M and S) and G_2 (between S and M) provide a safety gap before beginning with the next phase, allowing the cell to synthesize products necessary for the DNA-replication and to repair possible DNA-damages (reviewed by Blomen and Boonstra 2007). The correct progress of the cell cycle is controlled by a set of proteins, the cyclin-dependent protein kinases (CDK) and the cyclins, and feedback from downstream processes and the environment can hold these proteins off passing through checkpoints in the gap-phases and in the M-phase (reviewed by Malumbres and Barbacid 2009). If the cell fails to pass a checkpoint, cell cycle arrest is enforced. Notably, most signals resulting in cell cycle arrest act at the G_1 checkpoint, before entry into the S-phase. Cells then enter a resting state, G_0 (reviewed by Blomen and Boonstra 2007). Nine types of CDK-proteins, CDK 1 – CDK 9, and at least sixteen types of cyclins, cyclin A – cyclin T are known at present (reviewed by Blomen and Boonstra 2007). Different cyclin–CDK complexes are expressed in different phases of the cell cycle: cyclin D–CDK4/6 in G_1; cyclin E–CDK2 later in G_1 and at the G_1–S-phase transition; cyclin A–CDK2 during S, G_2 and M phase; and cyclin B–CDK1/2 at the G_2–M-phase transition (reviewed by Schwartz and Shah 2005).

The orderly transition between the cell cycle phases depends on the CDK-activity, which is regulated by several mechanisms, including activating and inactivating phosphorylations, binding to regulatory cyclin subunits, subcellular localization, and association with CDK inhibitors (CKI) as well as controlled degradation of regulatory subunits (reviewed by Lolli and Johnson 2005).

The cyclin-CDK-complex is a substrate for two protein kinases: the activating kinase MO15 (CDK7), which introduces a phosphate residue in the activating site of

the CDK molecule, and the inhibitory Wee1-protein, which phosphorylates a tyrosine residue close to the catalytic site of CDK, thereby decreasing the CDK-cyclin activity. This inhibitory phosphate group is then removed from the tyrosine residue by the phosphatase CDC25 (Figure 12) (Parker and Piwnica-Worms 1992; Shuttleworth 1995; Kellogg 2003).

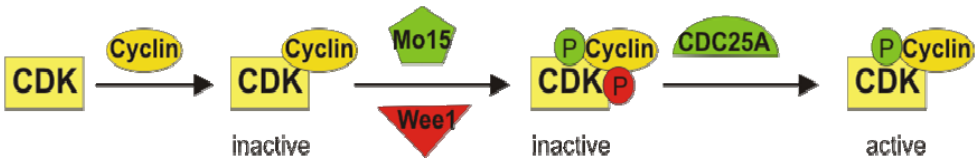

Figure 12. Progression through the cell division cycle is tightly controlled by complexes of cyclin dependent kinases (CDK) with cyclins. The activity of these complexes is regulated via phosphorylation by three different enzymes. First, the CDK-cyclin complex becomes a substrate for the kinase MO15, which introduces a phosphate residue on the activating site of the complex. In parallel, the kinase Wee1 decreases the activity of the CDK-cyclin complex by phosphorylating its catalytic site. This inhibitory phosphate residue is removed by the phosphatase CDC25A, thereby activating the CDK-cyclin complex. (Alberts et al 1994)

Three different CDC25 types, A, B and C, have been found in mammalian cells. The CDC25 subtypes are expressed and activated at different times during the cell cycle and have been shown to interact with different cyclin – CDK - complexes. CDC25A expression is required for S-phase entry and is induced in G_1 by the transcription factors c-Myc and E2F, while CDC25B and CDC25C act primarily at the G_2/M transition (reviewed by Kiyokawa and Ray 2008).

In a final step, the CDK-cyclin-complex adopts its correct conformation through another phosphorylation in its activation segment by the CDK-activating kinase, which is composed of cyclin H, MO15 and Mat1 (reviewed by Lolli and Johnson 2005).

CDK inhibitory proteins (CKI) belong to two families, which differ in their structure and CDK specificity. The first family, known as Cip/Kip (**k**inase **i**nhibitor **p**rotein) family, comprises $p21^{waf1/cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, which can bind to all G_1-cyclin/CDK complexes, though having a preference for CDK2. The second group includes the INK4 proteins (**in**hibitors of CD**K4**) $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, so-named because they specifically inhibit CDK4 and CDK6 (reviewed by Coqueret

2003). CKIs play an important role in the response of cells to growth-inhibitory signals, like induction of differentiation, p53 activation after DNA damage, contact-inhibition, senescence, etc. As expected from their capacity to block the progression of the cell cycle, CKIs are often inactivated in cancer (reviewed by Canepa et al. 2007).

As described above, checkpoints in the different phases of the cell cycle ensure the correct transcription of the genetic information. The first checkpoint to be passed through in G_1 is set by the retinoblastoma (Rb) tumor suppressor which, in its active, hypophosphorylated, state, forms inhibitory complexes with E2F-DP transcription factors, required for the start of the S-phase (reviewed by Schwartz and Shah 2005). Mitogens upregulate the expression of cyclin D which binds to CDK4 and CDK6 and activates them (reviewed by Blomen and Boonstra 2007). Increasing levels of cyclin D lead to the release of CDKs from their inhibitory complexes with Cip/Kip CKIs, and activation of the cyclin D-CDK4/6 pathway. Activated cyclin D-CDK4/6 partially phosphorylate and inactivate the Rb-protein, thereby releasing the E2F transcription factors, which can start with the transcription of genes, like cyclin E (Figure 13) (reviewed by Schwartz and Shah 2005). Cyclin E forms an active complex with CDK2 which completely hyperphosphorylates and inactivates Rb, thereby releasing and activating the E2F transcription factors. E2F now starts with the transcription of numerous S-phase proteins, like dihydrofolate-reductase, thymidine kinase, thymidylate synthase, DNA polymerase-α, CDK1, cyclin A, and E2F-1 itself (Dirks et al. 1998). At the same time, cyclin E-CDK2 phosphorylates and inactivates $p27^{KIP1}$, thereby counteracting the inhibitory influence of $p27^{KIP1}$ on cyclin A-CDK2 and allowing cyclin A-CDK2 to start the S phase (Muller et al. 1997). At this so-called restriction point, the cell cycle progresses independently of external stimuli, like growth factors, chemicals or metabolites. Remarkably, in tumors, characterized by their autonomous growth, this restriction point is frequently inactivated (reviewed by Malumbres and Barbacid 2009).

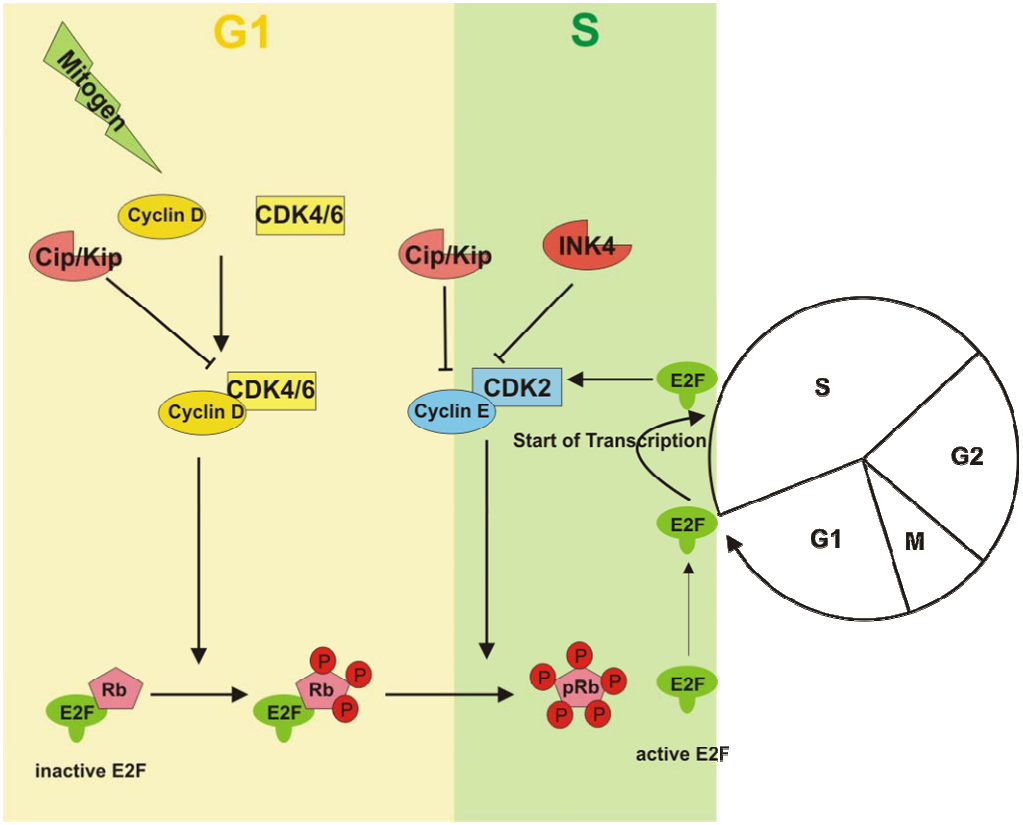

Figure 13. Molecular interplays at the transition from G1- to DNA-Synthesis (S) - phase of the cell cycle. In early G1, mitogens stimulate the formation of complexes of cyclin-dependent kinase 4 or 6 (CDK4/6) with cyclin D. The activity of this complex is negatively regulated by cyclin-dependent-kinase-inhibitory proteins (Cip). However, when a threshold level of cyclin D is reached, the CDK4/6-cyclin D- complex is not longer sequestered by Cip and can enhance the phosphorylation and thus inactivation of retinoblastoma protein (Rb), which thereby increasingly releases the E2F transcription factors. E2F starts with the transcription of cyclin E, which associates with CDK2 to an active complex. This complex is negatively regulated by two classes of CDK-inhibitors, the Cip and the inhibitors of CDK4 (INK4). Increasing levels of CDK2-cyclin E-complexes then completely hyperphosphorylate Rb resulting in total release of E2F and the passage through the G1-S-restriction point. From now on, cell cycle progression is independent of external mitogenic stimuli. (Kolch et al. 2002), modified.

The Rb pathway is also connected with another major tumor suppressor, p53. The p53 protein is short-lived, because an associated protein, Mdm2, induces its ubiquitination and degradation (reviewed by Soussi and Beroud 2001). Genetic damage results in the stabilization of p53 which thereupon triggers apoptosis or cell-cycle arrest by upregulation of the cyclin E-CDK-inhibitor $p21^{waf1/cip1}$ (reviewed by Kolch et al. 2002).

Remarkably, p53 upregulates its inhibitor Mdm2, creating a self-regulatory feedback loop that keeps the levels of p53 and Mdm2 in balance. The interaction between p53 and Mdm2 is counteracted by the protein ARF, thereby increasing the levels and stability of p53 (Figure 14) (reviewed by Soussi and Beroud 2001). ARF is an abbreviation for “alternative reading frame”, because it is encoded by the same genetic locus as the CKI $p16^{INK4}$, but is transcribed from a different promoter (reviewed by Gil and Peters 2006). Interestingly, ARF transcription is induced by E2F released from Rb (reviewed by Kolch et al. 2002). Therefore, it depends on the ratio of ARF to Mdm2 whether the cell passes the restriction point or not. Increased stabilization of p53 by ARF induces $p21^{waf/cip}$, which, via interaction with CDK2, prevents the inactivation of Rb by CDK2 thereby halting the cell at the G_1–S-phase boundary (reviewed by Soussi and Beroud 2001). Vice versa, the degradation of p53 enables the cell to overcome the restriction point. Therefore, loss or inactivation of the ARF- $p16^{INK4}$ gene affects both tumor suppressors, the Rb and p53 pathway (reviewed by Kolch et al. 2002).

In addition to its role in G1 arrest, p53 also enforces cell cycle arrest in other phases of the cell cycle, like in G2. Moreover, p53 plays an important role in DNA repair and senescence, where the cell can no longer enter the cell cycle and looses its ability to divide (reviewed by Blomen and Boonstra 2007).

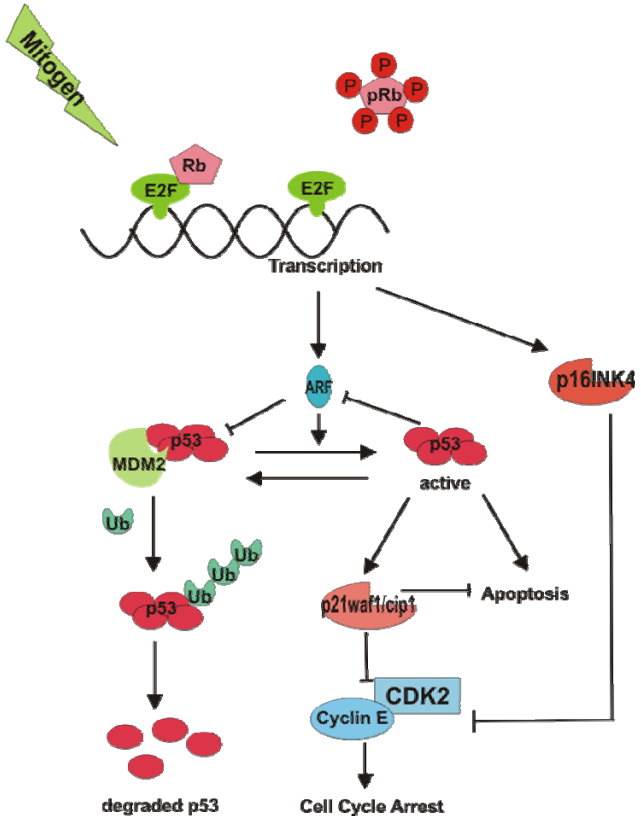

Figure 14. p53 pathway. Mitogenic hyperactivation results in stabilization of p53, which thereupon induces apoptosis or prevents cell cycle progression by upregulating the cyclin dependent kinase inhibitor $p21^{waf1/cip1}$. p53 is stabilized by ARF which prevents the interaction between p53 and its inhibitor Mdm2. Binding of Mdm2 to p53 increases the susceptibility of p53 to get ubiquitinylated (Ub) and degraded. ARF transcription is induced by E2F transcription factors, which are released when the retinoblastoma protein (Rb) is hyperphosphorylated (pRb). Interestingly, ARF is encoded by the same genetic locus as $p16^{INK4}$, which contributes to cell cycle arrest by inhibiting the activity of cyclin E-CDK2. (Soussi and Beroud 2001; Gil and Peters 2006).

3.4 Melatonin and Melatonin Receptors - Role in Cancer Development and Progression

3.4.1 Melatonin and its Actions on the Molecular Level

Melatonin, N-acetyl-5-methoxytryptamine, is a lipophilic hormone which is mainly produced and excreted in the pineal gland in a circadian rhythm during the night. The synthesis of melatonin from tryptophan in the pineal gland is controlled by the hypothalamic suprachiasmatic nucleus, which receives information from the retina on the environmental lightness (reviewed by Pandi-Perumal et al. 2008).

Because pineal melatonin production occurs during the dark and is suppressed by light and because melatonin is quickly removed from the circulation (half life approximately 30 min) (Brown et al. 1997), the time and duration of the melatonin peak reflects the environmental night period. Thus, melatonin is regarded a neuroendocrine mediator of the photoperiod (reviewed by Pandi-Perumal et al. 2008).

Besides its production in the pineal gland, synthesis of melatonin has been demonstrated in retina, gut, skin, bone marrow and lymphocytes, although these sites are only of local relevance because they do not influence the blood levels (Pandi-Perumal et al. 2008).

Many functions of melatonin in the body have been described, although its most important encompasses the regulation of circadian and seasonal rhythms. Melatonin reduces the plasma levels of gonadal estrogens via interaction with the hypothalamic pituitary axis (Cos et al. 2008). Other reported actions of melatonin include its involvement in the sleep-wake rhythm, aging process, bone metabolism, renal function, blood pressure control and regulation of the immune response (Brzezinski 1997; Ekmekcioglu 2006; Reiter et al. 2007).

These multiple effects of melatonin result from its action as free radical scavenger and its binding to membrane and nuclear receptors (Figure 15) (Reiter et al. 2007).

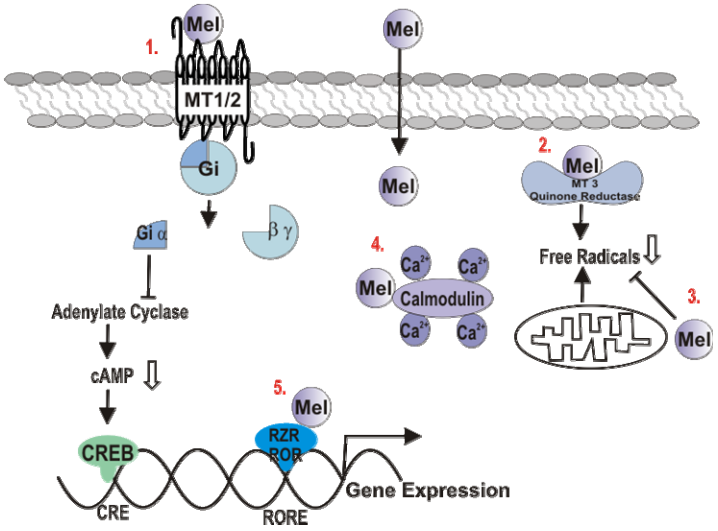

Figure 15. Sites of melatonin action at the cellular level. Binding of melatonin (Mel) to its 7-transmembrane MT1 or MT2 receptor leads to the cleavage of the α-subunit (G_i α) from the G_i protein linked to the receptor. This results in inhibition of adenylate cyclase activity and thus in decreased levels of cyclic adenosine monophosphate (cAMP) which is responsible for the phosphorylation and binding of the transcription factor cAMP response element binding protein (CREB) to its cAMP response element (CRE) in DNA (1). Due to its lipophilicity, melatonin passes the cell membrane easily. It prevents the formation of free radicals via interaction with MT3, a quinone reductase (2) and acts as scavenger of radicals (3). Other intracellular binding sites for melatonin include Ca^{2+}-calmodulin (4) and the nuclear receptors retinoid Z receptor (RZR) and retinoid orphan receptor (ROR). Upon activation by melatonin, RZR/ROR monomers bind to their RORα-response element (RORE) in DNA and start with gene expression (5). (Brzezinski 1997, modified).

A. Antioxidative Effects

Melatonin and its metabolites reduce oxidative damage by preventing the formation of free radicals and enhancing ATP production in the mitochondria which is required for the repair of cellular damage. Moreover, melatonin scavenges already formed free radicals (reviewed by Reiter et al. 2007).

B. Action through Membrane Receptors

Many effects of melatonin are mediated via its binding to its high-affinity seven-transmembrane receptors, MT1 and MT2. Due to their high homology at the amino acid level (about 55% overall and 70% within transmembrane domains), MT1 and MT2 form their own family which is part of the superfamily of G-protein coupled

receptors (GPCR) (reviewed by Jockers et al. 2008). Recently, another member of the melatonin receptor family, namely the melatonin related receptor GPR50, has been identified in human brain and peripheral tissues. However, this receptor is incapable of melatonin binding, and its natural ligand has not yet been identified (reviewed by Jockers et al. 2008). Also, MT1 and MT2 slightly differ in their affinity for melatonin, being lower for MT2. Melatonin binding and receptor mRNA levels underlie a circadian rhythm, and the expression levels of the receptor depend on the plasma melatonin concentration (reviewed by Pandi-Perumal et al. 2008).

Depending on the cellular context, diverse signaling cascades triggered by MT1 or MT2 are possible, involving in most cases the activation of both, pertussis toxin-sensitive G_i and –insensitive $G_{q/11}$ proteins that mediate the blockade of adenylate cyclase or the activation of phospholipase C-β (PLC-β). The inhibition of adenylate cyclase results in decreased levels of cyclic adenosine monophosphate (cAMP), in reduced activity of protein kinase A (PKA) and decreased phosphorylation of the nuclear transcription factor cAMP-responsive element binding protein (CREB) which is important for cAMP regulated gene expression and proliferation. On the other hand, the activation of PLC-β by the $\beta\gamma$-subunit of the G protein or by $G\alpha_q$ results in increased phosphorylation of the mitogen activated protein kinases (MAPK) MEK, ERK and JNK which activate transcription factors important for protein synthesis, cell growth, survival and motility (Figure 16) (reviewed by Wilkinson and Millar 2000; Lee et al. 2001; Goldsmith and Dhanasekaran 2007; Jockers et al. 2008). MT1 can also regulate ion channels, like large conductance calcium activated potassium channels or inward rectifier potassium channels (reviewed by Pandi-Perumal et al. 2008). Moreover, studies have shown that MT1, MT2 and GPR50 can form heterodimers which might also influence receptor signaling and trafficking (reviewed by Jockers et al. 2008). Therefore, MT receptors might use many diverse signaling pathways leading either to pro-differentiating or proliferative effects.

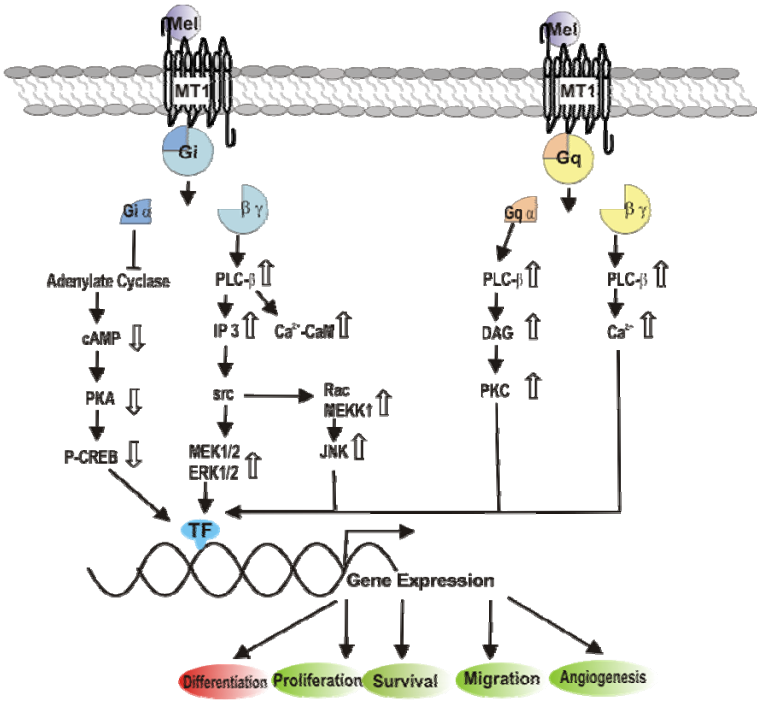

Figure 16. Overview on MT1-signaling pathways, according to: (Dubocovich and Masana 2003a, Jockers et al. 2008). MT1 is a seven-transmembrane receptor which couples to G_i or G_q proteins. Upon activation of the receptor, the heterotrimeric G-protein detaches and dissociates into its Gα- and G$\beta\gamma$- subunits. This leads to activation of several downstream signaling cascades: 1) $G_i\alpha$ decreases the levels of cyclic adenosine monophosphate (cAMP), thereby decreasing the activity of protein kinase A (PKA) on the phosphorylation of cAMP response element binding protein (CREB), a transcription factor which interacts with other transcription factors (TF), 2) phospholipase C-β (PLC-β) is activated by the $\beta\gamma$-subunits of G_i or G_q or by $G_q\alpha$. As consequence, the levels of inositol-triphosphate (IP3), and calcium-calmodulin (CaM) increase, leading to activation of the mitogen activated protein kinases MEK, ERK and JNK. Moreover, activation of PLC-β enhances the levels of diacylglycerol (DAG) and calcium which then lead to activation of protein kinase C (PKC). All mentioned signaling pathways influence diverse transcription factors (TF) and thereby the expression of genes involved in such different cell responses as differentiation, proliferation, cell survival, migration or angiogenesis.

MT1 and MT2 are expressed singly or together mainly in the central nervous system but also in many other sites in the body.

In the periphery, MT1 has been identified in the retina, coronary blood vessels and aorta, mammary gland epithelium, gastrointestinal tract, pancreas, gallbladder, liver, kidney, bladder, ovary, testis, prostate, skin, bone and in immune cells (reviewed by Ekmekcioglu 2006).

By contrast, MT2 is more restricted to the brain, but has been also found in lung, cardiac, aortic and coronary tissue, myometrium, immune cells, duodenum and adipocytes (reviewed by Ekmekcioglu 2006).

C. Action through Nuclear Receptors

In addition to its membrane receptors, melatonin acts also on related orphan receptors, located in the nucleus. These receptors belong to the RZR/ROR receptor subfamily, which includes three subtypes (α, β, γ) and four splicing variants of the α-subtype (RORα1, RORα2, RORα3, RZRα) (reviewed by Smirnov 2001). RZR/RORα is expressed in various organs, whereas RZRβ is specific for brain and retina (reviewed by Smirnov 2001). RORγ was identified in skeletal muscle, liver, kidney and adipocytes (reviewed by Smirnov 2001). Signaling through these receptors has been associated with the action of melatonin on immune cells and also with its activation of antioxidative enzymes (reviewed by Reiter et al. 2007).

D. Other intracellular Binding Sites for Melatonin

Melatonin interacts with intracellular molecules, like calmodulin, calreticulin and tubulin. Moreover, it was shown to prevent the formation of free radicals through its action on MT3, a quinone reductase (reviewed by Jockers et al. 2008; Pandi-Perumal et al. 2008).

3.4.2 Melatonin, MT1 and their Role in Breast Cancer

A. Evidence for a Role of Melatonin in Breast Cancer

It is well documented that melatonin plays a role in the initiation and progression of cancer. For example, women being constantly exposed to light at night, e.g. due to shift work, had an increased incidence of breast cancer which was accompanied by decreased urinary levels of melatonin and its metabolites (Schernhammer and Schulmeister 2004). Moreover, it was shown that this reduction in melatonin plasma and urinary levels was negatively associated with the ER-status (Tamarkin et al. 1982; Danforth et al. 1985; Bartsch et al. 1991). Further studies found an inverse relationship between nocturnal melatonin serum levels and the size of the primary breast tumor, and melatonin levels in breast cancer tissue correlated negatively with

the tumor differentiation grade (Bartsch et al. 1989; Maestroni and Conti 1996). In accordance with these investigations, studies conducted in animals showed that pinealectomy stimulates whereas melatonin administration inhibits tumor formation in rats treated with carcinogens (reviewed by Srinivasan et al. 2008).

B. Molecular Actions of Melatonin on Breast Cancer Cells

The oncostatic effect of melatonin on breast cancer results from its action as radical scavenger accompanied by activation of its membrane (MT1) and nuclear receptors (Ram et al. 2002; Grant et al. 2009).

Expression of MT1, but not of MT2, is found in human breast cancer cell lines and also in normal and malignant human breast tissue (Dillon et al. 2002; Lai et al. 2008).

Overexpression of MT1 significantly inhibited the proliferation of ER-positive MCF 7-cells, which were originally isolated from the pleural effusion of a woman with metastatic breast cancer (Yuan et al. 2002). Moreover, breast cancer cell lines have been shown to express nuclear RZR/RORα receptors, and high doses of the RZR/RORα agonist CGP-52608 were lethal for MCF-7 cells (Ram et al. 2002). Other oncostatic mechanisms of melatonin include its stimulating effect on immune cells and its anti-invasive and anti-metastatic actions (Table 3).

Effect	Suggested Mechanism	References
Inhibition of Proliferation and Induction of Apoptosis of Tumor Cells	inhibition of cyclin D1, increase in expression of p53 and $p21^{waf1/cip1}$	(Mediavilla et al. 1999; Cini et al. 2005)
	increase of degradation of calmodulin which is involved in the initiation of S- and M-phase of the cell cycle	(Blask et al. 2002)
	decrease in uptake of linoleic acid leads to decreased formation of 13-OH-Octadienoic Acid, which increases proliferation through activation of epidermal growth factor signaling	(Blask et al. 2004)
	increase in number of gap junctions leads to decreased proliferation rate	(Cos and Fernandez 2000)
	inhibition of telomerase activity which is important for cell survival	(Leon-Blanco et al. 2003)
	disruption of mitochondrial respiration and induction of apoptosis	(Scott et al. 2001)
	melatonin interacts with the tumor cell estrogen responsive pathway. It decreases i) circulating levels of gonadal estrogens via interaction with the hypothalamic pituitary axis, ii) the expression of ER-α and iii) the binding of the estradiol-ER complex to DNA, thereby preventing the transcription of ER regulated genes, e.g. of PR and cyclin D1. Moreover, melatonin downregulates the expression of genes responsible for the local synthesis or activation of estrogen	(Sanchez-Barcelo et al. 2003; Korkmaz et al. 2008)
Reduction of Vascular Growth	inhibition of the synthesis of endothelin 1 which is implicated in cell proliferation and neoangiogenesis	(Reiter 2004)
	melatonin induces apoptosis of human umbilical vein endothelial cells	(Cui et al. 2006)
Inhibition of Invasion and Metastasis	increase in expression of E-cadherin and β1-integrin	(Cos et al. 1998)
	melatonin induces a rearrangement of microfilaments and microtubules	(Ortiz-Lopez et al. 2009)
Immunoenhancement	increase in the activity of T- and B-lymphocytes, monocytes and natural killer cells	(Maestroni 1999)
	augmentation of secretion of cytokines, like interleukin-2, -6, -12 and interferon-γ	(Maestroni 1999)
Reduction of Oxidative Stress	melatonin acts as radical scavenger and reduces DNA damage	(Reiter et al. 1999)

Table 3. Mechanisms by which melatonin suppresses breast cancer proliferation.

Although studies on breast cancer cells in culture clearly showed an antiproliferative effect of melatonin via its interaction with MT1, a recent IHC study found higher MT1 levels in the tumor than in normal breast tissue (Dillon et al. 2002). Moreover, higher MT1 levels in the cancer specimens were significantly positively related to the tumor differentiation grade (Dillon et al. 2002). Interestingly, previous studies demonstrated that MT1 might be spontaneously active also in the absence of agonists and that this constitutive activity even occurs when the receptor is not experimentally overexpressed (Roka et al. 1999; Jockers et al. 2008). Since activation of MT1 may induce many different signaling cascades leading either to differentiation or proliferation (see chapter 3.4.1 and Figure 16), other functions of MT1 than those observed in cell culture seem therefore to be likely in breast cancer tissue.

3.4.3 Melatonin and Cell Cycle

The effects of melatonin on the cell cycle of breast cancer cells are associated with its inhibition of estrogen-induced cell proliferation.

Upon activation by estradiol, the estradiol-ER-α complex binds to their estrogen response element (ERE) in DNA and starts with the transcription of genes implicated in cell proliferation, like c-myc (reviewed by Renoir et al. 2008). The transcription factor c-myc itself then initiates the transcription of genes encoding for cell cycle regulators, such as cyclin D1 (reviewed by Butt et al. 2005). In addition to this indirect effect on cyclin D1 transcription, the estrogen-ER-α complex has been shown also to bind directly to a cAMP response element (CRE) on the cyclin D1 promoter, thereby upregulating cyclin D1 expression (reviewed by Renoir et al. 2008). Besides its direct action as transcription factor, ER-α regulates gene-expression via its binding to other transcription factors, like the Fos/Jun activating protein -1 complex (reviewed by Butt et al. 2005).

Melatonin abolishes these cell cycle promoting effects of estrogen signaling through multiple mechanisms. First, it reduces the levels of circulating estrogens,

and, second, it decreases the expression of ER-α (reviewed by Sanchez-Barcelo et al. 2005). Moreover, melatonin blocks the binding of estradiol-ER-α complexes to DNA and it also decreases the transcriptional activity of ER-α (reviewed by Sanchez-Barcelo et al. 2005). These effects were found to be mediated via MT1 (reviewed by Sanchez-Barcelo et al. 2005). Recently, melatonin was shown to inhibit the estradiol-induced upregulation of cyclin D1 transcription via interaction with the transcription factors ATF-2 and c-jun which bind to the cAMP response element in the cyclin D1 promoter (Cini et al. 2005).

Another mechanism through which melatonin delays cell cycle progression comprises the induction of p53 and $p21^{waf1}$ – expression (Cos et al. 2002). Moreover, a recent study showed that activation of MT1 by melatonin induces apoptosis which was associated with an increase in p53- but a decrease in Mdm2- levels (Cucina et al. 2008).

3.4.4 Melatonin and Cancer Stem Cells

A. Stem Cells and Cancer Stem Cells in the Breast

Adult stem/progenitor cells are a small dormant, long-lived and self-renewing population throughout the body, which have the capacity to differentiate into more specialized cell types (reviewed by Woodward et al. 2005). Depending on their differentiation potential, they may mature into cell types from all three germ layers (pluripotent), into several cells originating from one germ layer (multipotent) or only into one specialized cell type (unipotent) (reviewed by Wiese et al. 2004). It is thought that signals from the local environment (e.g. interaction of adhesion molecules with the extracellular matrix) maintain the stem cells in their quiescent and undifferentiated status, in the so called stem cell niche (reviewed by Woodward et al. 2005). When necessary, the stem cells proliferate to generate an exact copy of themselves (selfrenewal), thereby maintaining the stem cell pool, or they differentiate into cells with a limited self-renewal capacity or even a fully differentiated mature cell. This process is especially important after injury processes when the tissue has to be reconstituted. Progenitors may not only be restricted to one tissue but can also migrate to and settle down in other tissues, e.g. hematopoetic progenitors in skeletal

muscle or neuronal stem cells in the mammary gland (Asahara et al. 1997; Kawada and Ogawa 2001; Booth et al 2008).

In the mammary gland, stem cells are responsible for the rapid growth of the breast during puberty, but also for the changes during pregnancy and lactation (reviewed by Woodward et al. 2005). The presence of stem cells in breast tissue has been experimentally demonstrated by injection of breast progenitor cells, as defined by their capacity to efflux the dye Hoechst 33342, into cleared mammary fat pads of recipient mice which then developed intact and functional breast glands (Alvi et al. 2003).

During their long life-span, stem cells might accumulate mutations, giving rise to formation of cancer stem cells (reviewed by Yehiely et al. 2006). Indeed, there is increasing evidence that some cancers might be derived from stem cells. For example, molecular pathways involved in stem cell renewal, like Wnt, Notch, PTEN, are often deregulated in a number of tumors (reviewed by Ponti et al. 2006). Also, tumor cells show often similar properties like normal stem cells, e.g. low differentiation, self-renewal, migration and cytoprotection (e.g. overexpression of anti-apoptotic proteins, and overexpression of drug efflux pumps) (reviewed by Ponti et al. 2006). Tumor-initiating cancer stem cells were found in many hematological and solid malignancies, like breast cancer (reviewed by Gonzalez-Sarmiento and Perez-Losada 2008). Al-Haji and coworkers showed that a few hundreds of cells isolated from breast cancer patients and transplanted into mammary fat pads of immunodeficient mice could reconstitute the tumor from which they were derived (Al-Hajj et al. 2003). Remarkably, only tumor cells with high expression of the cell surface antigen CD44, together with no or low expression of CD24 showed this tumor-initiating potential. In an *in vitro* experiment performed by another group, $CD44^{+}CD24^{-}$ human breast cancer cells formed non-adherent spherical cell clusters, mammospheres, a property described previously only for normal mammary progenitors (Ponti et al. 2005). Interestingly, it has been suggested that the different molecular subtypes of breast cancer, like luminal-, HER2- or basal-like, might originate from breast progenitor cells at distinct stages of lineage differentiation, with luminal-like breast tumors arising from more differentiated cells and basal–like carcinomas from the most primitive stem cells (reviewed by Birnbaum et al. 2004). This is further supported by the fact, that basal-like breast carcinomas and mammary

stem cells show similar protein expression profiles, e.g. CK5, CK14, α6-integrin, vimentin and αB-crystallin (reviewed by Yehiely et al. 2006). With respect to their cytoprotective mechanisms and their capacity for self-renewal and unlimited proliferation, cancer stem cells have a crucial impact on the patient's further course of disease. Tumor stem cells might be resistant to established cytostatic therapy due to their low cell cycle kinetics, activation of DNA damage repair mechanisms, and their upregulation of drug efflux pumps (multidrug resistance transporter 1, MDR1; breast cancer resistance protein, BCRP) expelling chemotherapeutic agents out of the cancer cell, and may therefore survive and reconstitute the tumor, leading to cancer recurrence (reviewed by Eyler and Rich 2008).

B. Nestin – a Novel Marker for Cancer Stem Cells?

Nestin is a type VI intermediate filament protein of 240 kDa, which was first identified in **ne**uroepithelial **st**em cells (reviewed by Wiese et al. 2004). Intermediate filaments are polymeric components of the cytoskeleton and play also an important role in cell adhesion (reviewed by Omary et al. 2004). Like other intermediate filament proteins, the structure of nestin is composed of an α-helical central rod domain, but in contrast to the other intermediate filament proteins, the N-terminus is very short which makes it impossible for nestin to self-assemble. Therefore, nestin assembles with other intermediate filament proteins, like vimentin or desmin, or with microfilaments and microtubules via its long C-terminus (reviewed by Wiese et al. 2004).

Nestin is expressed by many cell types during development, but is downregulated upon differentiation. In differentiated cells it is replaced by other intermediate filament proteins such as glial acidic protein in astrocytes or desmin in muscle cells (Michalczyk and Ziman 2005). Nestin is highly expressed during early neurogenesis but is also found in proliferating and migrating cells in a variety of other fetal and embryonic tissues (reviewed by Wiese et al. 2004).

In adults, nestin-expressing cells are restricted to defined locations, where they may function as cellular reserve. These nestin-expressing cells have been found in the dermis, hair follicles, intestine, pancreas, bone marrow, testis, vascular endothelium, brain, central and peripheric nerve system, retina, heart, muscle and

other tissues, and show characteristic features of progenitor cells, such as multipotency, infinite proliferation, and self-renewal capacity (reviewed by Wiese et al. 2004; Kolar et al. 2007). Remarkably, expression of nestin is also found in myoepithelial cells lining the milk ducts in healthy breast tissue (Kolar et al. 2007). In premalignant lesions of the breast, like ductal or lobular carcinoma in situ, the nestin expression of myoepithelial cells persists, but is lost when the myoepithelial cells disappear and tumors become invasive (Kolar et al. 2007).

Concordant with the expression of nestin in progenitors, nestin is upregulated after tissue injury, e.g. in infarcted myocardium (Scobioala et al. 2008). The exact mechanisms or factors which regulate nestin expression are still unclear.

Nestin expression was also observed under malignant conditions in a cell population with characteristics of cancer stem cells which could differentiate into both, epithelial and endothelial cells (Bussolati et al. 2008). As already mentioned in chapter 3.4.A, breast carcinomas of the basal subtype show a transcriptional profile resembling that of mammary stem cells (Yehiely et al. 2006). In this regard, immunostaining of nestin was shown in tumor cells infiltrating the stroma of breast cancers of the ER-, PR-, HER2-triple negative (basal-like) subtype (Li et al. 2007; Parry et al. 2008). Moreover, in the study of Parry and coworkers, nestin expression was exclusively found in high grade, G3, tumors (Parry et al. 2008).

A correlation of nestin expression with dedifferentiation and tumor grade has been shown also for other tumors, e.g. of the central nervous system (Strojnik et al. 2007). Moreover, previous studies on U-373 MG human glioblastoma cells and on human prostate carcinoma cells showed an association between nestin expression and changes in cell shape and increased motility (Zhou and Skalli 2000; Kleeberger et al. 2007). Also, nestin is expressed in mitotically active cells, indicating a complex role for nestin in the regulation of intermediate filament assembly and thus in cell remodeling (Michalczyk and Ziman 2005).

Apart from breast cancer, upregulation of nestin expression has been found in many other tumors, e.g. melanoma, rhabdomyosarcoma, gastrointestinal stromal tumors, pancreatic adenocarcinoma, angiosarcoma, testicular stromal tumors and adrenocortical tumors (Kobayashi et al. 1998; Tsujimura et al. 2001; Lobo et al. 2004; Ackermann et al. 2005; Toti et al. 2005; Yang et al. 2008; Murphy et al. 2009). In

hormone independent prostate cancer, nestin expression was associated with formation of metastases and poor patient survival (Kleeberger et al. 2007).

Besides its expression in cancer stem cells, in neoplasias, nestin is also found in endothelial cells of newly formed small blood vessels (Mokry et al. 2004). Bussolati et al showed that nestin-expressing breast cancer progenitors might not only differentiate into epithelial but also into vascular endothelial cells leading to formation of capillaries (Bussolati et al. 2008). As already mentioned in chapter 3.2.2, angiogenesis is an important factor in the proliferation and metastasis of neoplasms. Indeed, a strong immunohistochemical staining of nestin was found in the capillary endothelium in breast tumors (Kolar et al. 2007).

C. Melatonin and MT1 - Impact on Stem Cells

The expression of MT1, together with nestin, in the murine neural stem cell line C17.2 was recently reported (Niles et al. 2004). Neural stem cells are multipotent which means that they can differentiate into several types of neural cells, like neurons, astrocytes or oligodendrocytes. Of special interest for this thesis, another study showed that neural stem cells can migrate to and reside in a new microenvironment and can take over the function of mammary progenitors (Booth et al. 2008). Moreover, in C17.2 neuronal stem cells, it was found that activation of MT1 by melatonin induces the expression of the glial cell-line derived neurotrophic factor (GDNF), and that this effect can be inhibited by the adenylate cyclase activator forskolin, followed by induction of differentiation processes (Niles et al. 2004). GDNF enhances proliferation, survival and migration of neurons in brain, spinal cord and periphery via its binding to glycosyl phosphatidylinositol-linked GDNF family receptor-α (GFR-α), which recruits the receptor tyrosine kinase RET (Esseghir et al. 2007). Remarkably, a recent study showed the expression of GDNF, RET and GFR-α also in breast cancer specimens, and high GFR-α-mRNA levels were associated with lymphovascular invasion and lymph node metastasis (Esseghir et al. 2007). Taken together, melatonin might induce proliferation and/or enhance survival of progenitors or already differentiated cells through upregulation of GDNF expression in (neuronal) stem cells expressing MT1. This might be of relevance in breast carcinogenesis since neural stem cells may settle down also in peripheral tissues like the breast.

3.4.5 Metabolism of Melatonin

The efficacy of melatonin's action is limited by metabolizing enzymes which decrease the required intracellular concentration of melatonin.

A. Hepatic Metabolism

Upon release into the blood stream, melatonin is rapidly cleared in a single passage through the liver, where it is mainly metabolized to 6-OH-melatonin by the cytochrome P450 enzymes (CYP) 1A2, 1A1 and, to a minor extent, also by CYP2C19 (Ma et al. 2005). Around 70% of 6-OH-melatonin is then conjugated with sulfate residues while only 6% is glucuronidated (Cardinali 1981). This conjugation reaction enhances the substrate's hydrophilicity and thereby its excretion into urine. The conjugation of 6-OH-melatonin to sulfate is mainly catalyzed by the enzyme sulfotransferase (SULT) 1A1 which shows a high affinity particularly to 6-OH-melatonin, as compared to its parent compound melatonin (Aust et al. 2005). In addition, formation of 6-OH-melatonin sulfate from 6-OH-melatonin might be also partially catalyzed by SULT1A3, although the main role of this enzyme lies in the sulfate-conjugation of the monoamines dopamine and serotonin, the precursor in melatonin synthesis (Honma et al. 2001).

Remarkably, the urine levels of melatonin metabolites are exactly reflective of the plasma melatonin levels (Schernhammer et al. 2008). Therefore, the quantification of urine melatonin metabolites gives detailed information about circulating melatonin levels.

B. Metabolism of Melatonin in Breast Cells

Many studies have assessed the expression of CYPs and SULTs in breast cancer cell lines and also in tissue specimens. For example, the expression of CYP1A1 mRNA and protein was demonstrated in tumor and adjacent histologically normal breast tissue by quantitative reverse transcription polymerase chain reaction and immunohistochemistry, respectively (Modugno et al. 2003; Haas et al. 2006). Also, the expression of SULT1A1- and 1A3- mRNA has been recently shown in breast tissue (Aust et al. 2005a).

However, only limited data with respect to the metabolism of melatonin in breast tissue exist. To date, only one study examined the role of SULT1A1 in the breast cancer cell lines MCF-7 (ER-α expressing) and MDA-MB231 (ER-α negative) (Aust et al. 2005). In this study it was shown that the mRNA levels of SULT1A1 were lower in the ER-α negative MDA-MB231 cells as compared to the ER-α expressing MCF-7 cells, and that significantly more 6-OH-melatonin was sulfonated after stable transfection of MDA-MB231 cells with SULT1A1. This suggests an important role for SULT1A1 in the metabolism of melatonin in breast cancer. Another important point shown in this study was that sulfonation of parent melatonin was with only 1% very low as compared to that of its metabolite 6-OH-melatonin. Therefore, the expression and activity of CYP1A1 is a prerequisite for the metabolism of melatonin to 6-OH-melatonin sulfate (Figure 17).

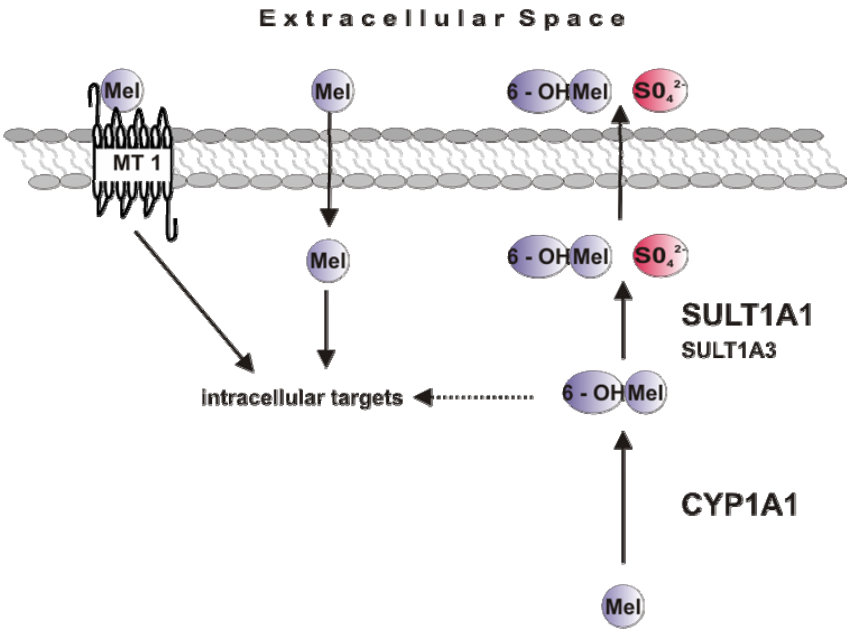

Figure 17. Metabolism of melatonin in breast cells. The cytochrome P450 enzyme CYP1A1 catalyzes the oxidation of melatonin (Mel) to 6-hydroxymelatonin (6-OH-Mel), which shows also anti-oxidant properties and which also binds to MT1, although to a lesser extent than melatonin. The sulfotransferases SULT1A1, and partially also SULT1A3 mediate the sulfonation of 6-OH-Mel to sulfate residues, which enhances its hydrophilicity. The resulting metabolite, 6-hydroxymelatonin sulfate (6-OH-Mel-$SO4^{2-}$), is then transported out of the cell. (Honma et al. 2001; Dubocovich et al. 2003; Tan et al. 2007).

3.5 References

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4 AIMS OF THE THESIS

Breast cancer is worldwide the most common cancer form among women. Although the mortality rates have declined in the past two decades due to earlier detection and better treatment options, the biomarkers currently in use have failed to predict the course of disease in individual cases. This suggests that there are still unknown factors playing a role in the development and progression of breast cancer, and their investigation should therefore provide additional prognostic information on the patient's particular course of disease and his response to a certain therapy.

It is already known that carcinogenesis and tumor growth are the result of uncontrolled cell division. The replication and division of a cell is coordinated by complexes of cyclins with cyclin-dependent kinases and their inhibitors. Failures in this interplay of the cell cycle regulators might lead to uncontrolled cell division and are associated with initiation and progression of cancer.

It has been shown that melatonin, bound to its receptor MT1, is able to suppress proliferation of breast cancer cells by inhibition of cyclin D1. The efficacy of melatonin action is determined by its intracellular concentration and therefore by its biotransformation and extrusion from the cell.

The intermediate filament protein nestin, a marker of stem/progenitor cells, is upregulated in highly aggressive basal-like breast cancer. In neuronal stem cells expressing both nestin and MT1, activation of MT1 led to enhanced cell proliferation, instead of inhibiting it, and to suppression of apoptosis.

This thesis proposes to test a panel of molecules for their relevance and appropriateness as novel biomarkers for predicting relapse and/or cancer-associated death in breast cancer.

i) It will be examined whether the expression level of any of the cell cycle regulators (cyclin D1, cyclin E, cyclin A, their activating phosphatase CDC25A, the cyclin-dependent kinase inhibitors $p16^{INK4a}$, $p21^{waf1/cip1}$, $p27^{kip1}$ and retinoblastoma protein) correlates with the patient's risk of relapse or cancer-associated death.

ii) The expression of the melatonin receptor MT1 and of the melatonin-metabolizing enzymes (CYP1A1, CYP1A2, SULT1A1, SULT1A3) will be analyzed in order to understand the possible relevance of melatonin in the pathogenesis and/or therapy of breast cancer. Also, a possible correlation between MT1 and Cyclin D1 expression will be examined.

iii) It will be investigated which breast cells express both nestin and MT1, and whether coexpression of MT1 with nestin is associated with tumor stage or the patient's risk of relapse.

The data obtained in this thesis will improve our molecular understanding of the early steps in carcinogenesis and also of tumor progression, and might give additional prognostic information regarding the patient's risk of relapse and cancer-associated death. Moreover, the assessment of melatonin-metabolizing enzymes in breast tissue might be of relevance to identify patients likely to benefit from melatonin as novel adjuvant drug with antiproliferative and immunoenhancing properties in breast cancer therapy.

5 RESULTS

Original Papers and Manuscripts

Cell Cycle Dysregulation influences Survival in High Risk Breast Cancer Patients

Königsberg R, Rögelsperger O, Jäger W, Thalhammer T, Klimpfinger M, De Santis M, Hudec M and Dittrich C.

Cancer Invest 2008, 26: 734-40.

Coexpression of the Melatonin Receptor 1 and Nestin in Human Breast Cancer Specimens

Rögelsperger O, Ekmekcioglu C, Jäger W, Klimpfinger M, Königsberg R, Krenbek D, Sellner F and Thalhammer T.

J Pineal Res 2009, 46: 422-32.

Expression of Melatonin - Metabolizing Enzymes and Cyclin D1 in Human Breast Cancer

Rögelsperger O, Wlcek K, Ekmekcioglu C, Svoboda M, Königsberg R, Klimpfinger M, Jäger W and Thalhammer T.

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Robert Königsberg, **Olga Rögelsperger**, Walter Jäger,
Theresia Thalhammer, Martin Klimpfinger, Maria De Santis,
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ORIGINAL ARTICLE
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Cell Cycle Dysregulation Influences Survival in High Risk Breast Cancer Patients

Robert Königsberg,[1] Olga Rögelsperger,[2] Walter Jäger,[3] Theresia Thalhammer,[2] Martin Klimpfinger,[4] Maria De Santis,[1] Marcus Hudec,[5] and Christian Dittrich[1]

Kaiser Franz Josef-Spital, Third Medical Department, Centre for Oncology and Hematology, CEADDP, Applied Cancer Research, Institution for Translational Research Vienna (ACR-ITR VIEnna), and Ludwig Boltzmann-Institute for Applied Cancer Research (LBI-ACR VIEnna), Vienna, Austria.[1]
Center for Physiology and Pathophysiology, Medical University of Vienna, Vienna, Austria.[2]
Department of Clinical Pharmacy and Diagnostics, University of Vienna, Vienna, Austria.[3]
Institue of Bacteriology and Pathology, Kaiser Franz Josef-Spital, Vienna, Austria.[4]
Department of Scientific Computing, University of Vienna, Vienna, Austria.[5]

ABSTRACT

Background: **Cell cycle progression is regulated by cyclin dependent kinases (cdk) and cdk inhibitors. Recent immunohistological studies suggested that dysregulation of cyclin A, cyclin D, cyclin E, $p16^{ink4}$, $p21^{waf1/cip1}$, and $p27^{kip1}$ are of prognostic value in patients with breast cancer. Our study represents the first comprehensive immunohistochemical cell cycle marker analysis for cdc25A, cyclin A, cyclin D, cyclin E, $p16^{ink4}$, $p21^{waf1/cip1}$, $p27^{kip1}$, and pRb in tumor tissue and adjacent benign breast tissue from 69 primarily untreated breast cancer patients.** ***Methods:*** **Immunhistochemistry using primary monoclonal antibodies to detect cdc 25A, cyclin A, cyclin D, cyclin E, $p16^{ink4}$, $p21^{waf1/cip1}$, $p27^{kip1}$, and pRb has been performed.** ***Results:*** **Sixty-nine patients with untreated, invasive breast cancer ($n = 69$) were divided into a low/ intermediate and a high risk group according to the St. Gallen 2005 consensus conference. High risk patients ($n = 22$) had a significantly ($p = 0.003$) shorter mean and median survival (282.85 weeks; 383.0 weeks, respectively) than low/intermediate risk patients (375.41 weeks; not reached yet, respectively). A subgroup of high risk breast cancer patients characterized in addition by overexpression of cdc25A, cyclin A, cyclin E, $p16^{ink4a}$, and $p27^{kip1}$ experienced a shortened mean survival of 222.03, 235.71, 257.25, 239.18, and 261.94 weeks, respectively. Regarding benign breast tissue adjacent to breast cancer tissue, 59.4% of the patients investigated overexpressed cdc25A, 23.2% overexpressed pRb, and 63.2% exerted dysregulation of $p27^{kip1}$ while they proved to be negative for immunohistochemical staining regarding all other markers tested.** ***Conclusion:*** **The immunohistological analyses of cdc25A, cyclin A, cyclin E, $p16^{ink4a}$, and $p27^{kip1}$ have the potential for further refining the risk assessment in patients with untreated breast cancer who belong to the high risk category defined according to the St. Gallen 2005 consensus conference. These cell cycle markers define a subgroup of high risk patients with even higher risk of metastazation and shortened survival. For confirmation a prospective study using standardized laboratory procedures in a larger population is needed.**

Keywords: High risk breast cancer patients, cdc25A, cyclin A, cyclin E, $p16^{ink4a}$, $p27^{kip1}$
Correspondence to:
Robert Königsburg, Kaiser Franz Josef-Spital, Third Medical Department, Centre for Oncology and Hematology, CEADDP, Applied Cancer Research, Institution for Translational Research Vienna (ACR-ITR VIEnna), and Ludwig Boltzmann-Institute for Applied Cancer Research (LBI-ACR VIEnna)
e-mail: rkoenigsberg@yahoo.de

INTRODUCTION

Uncontrolled cell proliferation is a prominent feature in tumorigenesis. Inappropriate activation of cyclin dependent kinases (cdks) can result in loss of checkpoint control and thus abnormal cellular proliferation. Cells traverse the cell cycle in several tightly controlled phases, G1, S, G2, and M (1). Cell cycle progression is a complex process mediated by cdks. Cdks, a class of serine/threonine kinases, consist of catalytic subunits that form complexes with proteins known as cyclins. Actually, there are nine cdks, cdk1–cdk9, and 15 cyclins, cyclin A–cyclin T known (2, 3). Cdk1 (cdc2) binds cyclin A and B to modulate G2–M transition, cdk2 binds cyclins A, D, and E to drive the G1–S transition and S-phase, cdk4 and cdk6 bind cyclin D to promote cell cycle progression through G1, and cdk7 binds cyclin H to activate other cyclin/cdk complexes (4). The level of activity of the holoenzymes formed by the cyclins and their cognate cdk catalytic subunits is regulated by distinct molecular mechanisms, which include phosphorylation and dephosphorylation of the kinase subunit (5). Cyclin expression varies during the cell cycle. Cdk activation depends upon removal of inhibitory Thr/Tyr phosphorylation by members of the cdc25 phosphatase family. Cdc25A expression is required for S-phase entry and is induced in G1 by myc and E2F (6). The E2F family of transcription factors, which control the expression of genes needed for cell cycle progression, is controlled by Rb family proteins, pRb and p107 (7). In addition, association with specific regulatory proteins (8) and interaction with specific cdk inhibitors are also of importance in the regulation of cdk activity. Two different types of cdk inhibitors can be distinguished (9, 10). First, inhibitors of cdk4 (INK4) comprise p16, p15, p18, and p19, and inhibit specifically cyclin D-associated kinases. The second type encloses the kinase inhibitor protein family (KIP) p21, p27, and p57, that bind and inhibit the activity of cyclin E/cdk2 and cyclin A/cdk2 complexes (3, 11, 12).

The prognostic value of individual cyclins and endogenous physiologic cyclin inhibitors has yet to be validated before entering routine clinical practice in breast cancer patients. It remains unclear whether the determination of additional tumor biological factors, including cdc25A, cyclin A, cyclin D, cyclin E, $p16^{ink4}$, $p21^{waf1/cip1}$, p27, and pRb, provides additional information on survival and response for the treatment of breast cancer patients with endocrine and chemotherapy.

The aim of this investigation was to conduct a complete cell cycle regulation analysis in a collective of untreated breast cancer patients using the above-mentioned parameters. In addition, we wanted to determine whether cyclins/cyclin inhibitors may be related to risk categories for patients with early breast cancer as defined by the St. Gallen 2005 consensus panel, which classifies breast cancer patients into low-, intermediate-, and high-risk categories (13), with regard to the influence of these parameters on overall and disease-free survival.

PATIENTS AND METHODS

Patients

Patients were informed of the investigational nature of this analysis and had given written informed consent in accordance with institutional and federal guidelines. The study protocol was approved by the ethical committee of the institution.

Sixty-nine consecutive patients with untreated, invasive breast cancer of whom all of the below detailed characteristics were available and parameters could be determined were included in this single hospital-based, retrospective analysis. The patients were diagnosed with breast cancer at the Kaiser Franz Josef-Spital, Vienna, Austria between 1998 and 2003. Their median follow-up was 62 months (range 25.0–92.0 months).

Patients were classified by age, tumor size, tumor grade, number of involved lymph nodes, type of surgery, and hormone receptor status. Lymph node dissection was performed in all patients but one, from whom nodal status was not available. Fifty-five patients (79.7%) had invasive ductal carcinoma, 9 (13.0%) patients had invasive lobular carcinoma, and 5 (7.3%) patients had other histological features. Further pertinent demographic features as well as their corresponding prognostic associations with regard to survival are summarized in Table 1.

Fifteen (21.7%) patients underwent modified radical mastectomy, and 54 (78.3%) underwent conservative breast surgery followed by radiation therapy. All patients received adjuvant chemotherapy containing anthracyclins. Estrogen receptor positive patients were additionally treated with tamoxifen or letrozol.

Seventeen (24.6%) of the 69 patients relapsed. Fourteen (20.3%) patients developed distant metastases at multiple sites involving lung (64.3%), bone (42.8%), liver (42.8%), brain (28.6%), and skin (14.3%). These 14 patients with distant metastases received polychemotherapy; among them 8 HER2/neu positive patients received in addition trastuzumab. Three (4.3%) patients had local recurrence. They underwent modified radical mastectomy but received no further chemotherapy. Overall, twelve (17.4%) patients died of breast cancer during follow-up.

Immunohistochemical analyses

Tumor specimens were obtained at the time of surgery before any chemo-, hormone- and radiotherapy. Formalin-fixed, paraffin embedded blocks from the primary breast lesions were used for all immunostaining procedures. One micrometer thick sections were cut and mounted to coated slides. After antigen retrieval by the microwave technique the immunostaining was processed in an automated staining machine (Techmate 500; DAKO, Gastrup, Denmark) following the operating manual. All series included negative and positive controls.

Table 2 summarizes the supply of the primary monoclonal antibodies to detect cdc 25A, cyclin A, cyclin D, cyclin E, $p16^{ink4}$, $p21^{waf1/cip1}$, $p27^{kip1}$, and pRb.

Table 1. Patient Characteristics and the Corresponding Prognostic Association with Regard to Survival in form of Univariate Analyses According to the St. Gallen 2005 Consensus Conference (12).

Factor	Cases ($n = 69$)		p value	RR	95% CI
Age, years			0.98	1.00	0.94–1.06
< 35	2	(2.9%)			
≥ 35	67	(97.1%)			
Pathologic tumor size			0.66	0.90	0.56–1.43
pT1	38	(55.1%)			
pT2	26	(37.7%)			
pT3	4	(5.8%)			
pT4	1	(1.4%)			
Lymph node status			< 0.001	1.24	1.19–1.38
Negative	40	(58.1%)			
Positive: 1–3 lymph nodes	13	(18.8%)			
Positive: 4–10 lymph nodes	11	(15.9%)			
Positive: $>$ 10 lymph nodes	5	(7.2%)			
Tumor Grade [1]			0.15	1.56	0.85–2.92
G1	4	(5.8%)			
G2	33	(47.8%)			
G3	32	(46.4%)			
Hormone receptor status					
Estrogen–receptor			0.21	0.48	0.16–1.51
ER-negative	25	(36.2%)			
ER-positive	44	(63.8%)			
Progesterone-receptor			0.22	0.45	0.12–1.65
PgR-negative	41	(59.4%)			
PgR-positive	28	(40.6%)			
KHER2/neu-receptor[2]			0.02	3.94	1.19–13.01
HER2/neu-negative[2]	54	(78.3%)			
HER2/neu-positive	15	(21.7%)			

ER = estrogen receptor; PgR = progesterone receptor.
[1]Grading was based on the recommendations of Elston and Ellis (15).
[2]HER/2neu-gene was determined as described by Isola et al. (16).

Evaluation and scoring of breast cancer tissues in the 69 patients

The slides were independently examined by two observers (RK and OR) who were blinded to clinical data before evaluation. In case of discrepant scoring, a final consensus was reached by using a double-headed microscope.

Cells were considered positive for cdc25A, cyclin A, cyclin D, cyclin E, $p21^{waf1/cip1}$, $p27^{kip1}$, and pRb only when distinct nuclear staining was identified. For staining of $p16^{ink4a}$ both nuclear and cytoplasmatic staining were considered as positive staining pattern.

The percentage of immunoreactive nuclei was evaluated by scanning the whole section at medium magnification and counting at least 500 cells using 10 high power fields (HPF; 40x objective lens; diameter of microscopic field: 0.45 mm).

Staining intensities were classified as: no staining (0), weak positive (1), moderate positive (2), and strong positive (3). The immune reactive score (IRS) was evaluated by multiplying the percentage of immunoreactive nuclei and the staining

Table 2. Monoclonal Antibodies Used for Immunohistochemistry

Antibody	Clone	Dilution	Incubation time	Positive Control
cdc25A ([1]LabVision)	DCS-120+ DCS-121	1:100	30 min	Tonsil tissue
cyclinA ([1]LabVision)	CYA06	1:20	60 min	Tonsil tissue
cyclinD1 ([2]DAKO)	DCS-6	1:80	30 min	Rectum epithelium
cyclinE ([1]LabVision)	CYE05	1:30	90 min	Placenta tissue
$p16^{ink4a}$ ([2]DAKO)	E6H4	1:25	30 min	Tonsil tissue
$p21^{waf1/cip1}$([2]DAKO)	SX118	1:25	30 min	Gut mucosa
$p27^{kip1}$ ([2]DAKO)	SX53G8	1:250	30 min	Tonsil tissue
pRb ([2]DAKO)	Rb1	1:50	30 min	Tonsil tissue

[1]LabVision, Westinghouse Dr. Fremont, California,, USA
[2]DAKO, Gostrup, Denmark

intensities according to the method of Remmele and Stegner (14) considering an IRS of 3 to 12 points as positive and an IRS below 3 points as negative.

For the $p27^{kip1}$ evaluation a different scoring system was used. In accordance to previously published data (15, 16) indicating associations between reduced $p27^{kip1}$ levels and shortened survival, $p27^{kip1}$ expression was classified as low (<50% of immuoreactive cells) or high (≥50% of immuoreactive cells).

Evaluation and scoring of normal breast tissues in the 69 patients

As first step, two independent observers (RK and OR) confirmed the benign nature of breast tissue being adjacent to the breast cancer tissue by microscopic control of the hematoxylin and eosin stained specimens. As next step, normal breast tissue from 20 patients was stained with all eight antibodies as described above (Table 2). If prominent immune reaction with a particular antibody was observed, samples from the remaining 49 patients were stained consecutively. Evaluation and scoring process were performed as described above.

Statistical analysis

Clinical, pathological, and molecular data were collected in a mutually blinded fashion from 1998 to 2003. Survival rates and follow-up data were calculated from the date of diagnosis until the time of the last clinical appointment or the date of proved breast cancer associated death.

The statistical evaluation of results included Fisher's exact test and X^2 test for univariate analyses. Survival time was calculated using Kaplan-Meier estimates, and differences between survival curves were analyzed by means of the Breslow and log-rank tests. The goal of the multivariate analyses was to evaluate the associations between each immunohistochemical marker and the new risk categories as defined by the St. Gallen consensus panel 2005 (13). Multivariate analyses of the survival time results were performed using the Cox proportional hazards regression model. The endpoints used in this study were overall survival and disease free survival. All statistical calculations were performed using the SPSS 14.0 statistical software (Chicago, Illinois, USA).

RESULTS

Immunohistochemical results of breast cancer tissue in the 69 breast cancer patients

Thirty-six (52%) of primary breast cancers were positive for cdc25A immunostaining; twenty (29.0%) patients were cyclin A positive; 40 (58%) patients were positive for cyclin D; 12 (17.4%) patients were positive for cyclin E; 25 (36.2%) patients were positive for $p21^{waf1/cip}$; 16 (23.2%) patients were positive for $p16^{ink4a}$; and 30 (43.5%) patients were positive for pRb. Regarding $p27^{kip1}$ 46 (66.7%) patients had low (<50%) expression, and 23 (33.3%) patients had 50% or more of immunostain reacting cells.

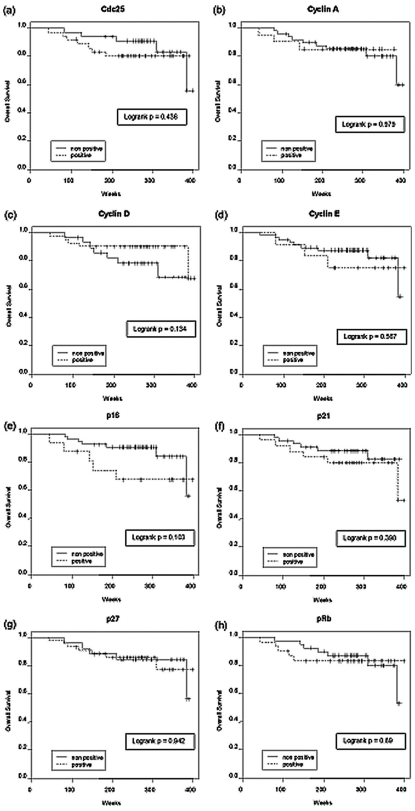

Figure 1. Univariate analyses of survival in 69 breast cancer patients with and without overexpression of a) cdc25A, b) cyclin A, c) cyclin D, d) cyclin E, e) p16ink4, f) p21waf1/cip1, g) p27 and h) pRb.

In univariate analyses, no statistically significant association could be observed between cdc25A, cyclin A, cyclin D, cyclin E, $p16^{ink4a}$, $p21^{waf1/cip1}$, $p27^{kip1}$, pRb, and overall survival (Fig. 1) as well as disease-free survival (data not shown).

Immunohistochemical results of normal breast tissue in the 69 breast cancer patients

In the case of cyclin A, cyclin D, cyclin E, $p16^{ink4a}$, and $p21^{waf1/cip1}$, no staining at all could be observed in the first 20 patients with benign breast tissue. Thus, the benign breast tissue of the remaining 49 patients was not stained.

Regarding cdc25A, $p27^{kip1}$, and pRb, positive staining was prominent. Fifty-two (75%) of the 69 patients' samples were stained with cdc25A, and 41 (59.4%) among them were immunohistochemically positive. Fifty-seven (82.6%) patients were stained with $p27^{kip1}$, and 36 (63.2%) among them had less than 50% of immunostain reacting cells; in 8 patients, no staining at all was present. Fifty-eight (84.1%) patients were stained with pRb, among them 16 (23.2%) were immunohistochemically positive. In univariate analyses, no statistically significant association could be observed between overall survival and cdc25A ($p = 0.82$), $p27^{kip1}$ ($p = 0.66$),

pRb ($p = 0.28$), or disease-free survival and cdc25A ($p = 0.15$), $p27^{kip1}$ ($p = 0.80$), and pRb ($p = 0.15$) (data not shown).

Univariate and multivariate survival analyses according to cdc25A, cyclin A, cyclin D, cyclin E, $p21^{waf1/cip1}$, $p16^{ink4a}$, $p27^{kip1}$, pRb, and defined risk groups

According to the St. Gallen 2005 consensus panel criteria (13), we formed a low, an intermediate, and a high-risk group from the 69 patients analyzed. Because the low risk group comprised only 3 (4.3%) patients, we pooled the patients of the low and intermediate risk group. Thus, the low/intermediate risk group comprised 47 (68.1%) patients, and the high risk group 22 (31.9%) patients.

Overall survival and disease-free survival of the 69 patients were calculated in an intent-to-treat manner from the time of diagnosis onwards (Fig. 2). The mean and the median overall survival of patients in the high risk group was significantly shorter than for the better risk patients ($p = 0.003$). Low/intermediate risk patients had a mean overall survival of 375.41 weeks (CI; 359.34–391.47). The median overall survival was not reached during the entire follow-up period of median 62 months yet. High risk patients had a mean overall survival of 282.85 weeks (CI; 221.87–343.83) and a median overall survival of 383.00 weeks (CI; 37.75–728.24). Regarding disease-free survival, low/intermediate risk patients had a mean disease-free survival of 359.78 weeks (CI; 336.83–382.74), whereas their median disease-free survival was not reached. High risk patients had a mean disease-free survival of 229.63 weeks (CI; 164.19–295.07) and a median disease-free survival of 240.00 weeks (CI; 16.33–463.66) ($p < 0.001$). Table 3 summarizes mean overall survival durations of the low/intermediate and the high risk patient group separately according to the individual samples' cell cycle marker expression analysed in univariate analyses in our study. There was an obvious tendency towards shortened overall survival in high risk patients expressing cdc25A, cyclin A, cyclin E, $p16^{ink4a}$, and $p27^{kip1}$ with respective mean survival times of 222.03, 235.71, 257.25, 239.18, and 261.94 weeks.

However, when the traditional clinico-pathological combined risk factors of the St. Gallen 2005 consensus panel and the cell cycle markers investigated in this study (cdc25A, cyclin A, cyclin D, cyclin E, $p21^{waf1/cip1}$, $p16^{ink4a}$, $p27^{kip1}$, pRb) were all included in a multivariate Cox regression analysis, only the established combined risk factors positive lymphnode status and positive HER2/neu-receptor status of the St. Gallen 2005 consensus panel were found to have a significant ($p < 0.005$) impact on breast cancer specific survival.

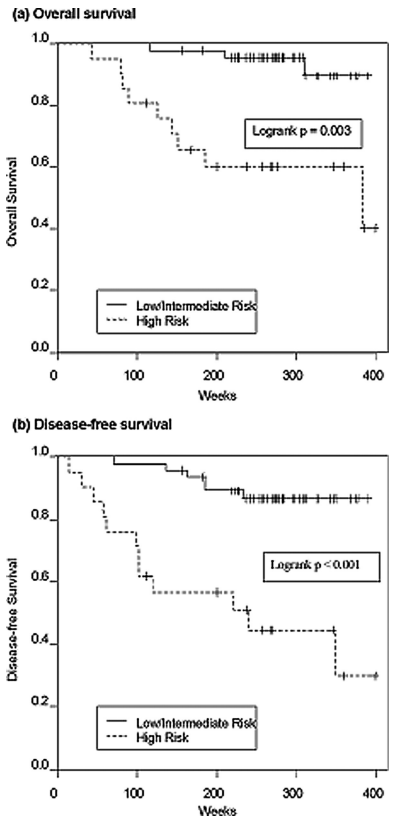

Figure 2. Overall survival and disease-free survival in 69 patients with primary breast cancer according to the St. Gallen 2005 Consensus risk categories.

DISCUSSION

Cdk's and their inhibitors represent biologically important factors which may dispose on prognostic potential for future risk assessment in breast cancer patients. Several research groups have investigated the link between immunohistochemically examined cell cycle regulators and prognosis, but definite molecular markers describing the individual risk for patients at high risk to relapse more accurately are still insufficient or missing (17–19).

Table 3. Overall Survival of Low/ Intermediate Risk Versus High Risk Patients (n = 69) Expressing cdc25A, Cyclin A, Cyclin D, Cyclin E, $p21^{waf1/cip1}$, $p16^{ink4a}$, $p27^{kip1}$ and pRb Separately in Univariate Analyses.

	Survival[1] (weeks) in low/ intermidiate risk patients	Survival[1] (weeks) in high risk patients
cdc25A	378.58; CI[2] (356.00–400.49)	222.03; CI (152.27–291.79)
cyclin A	334.60; CI (288.79–380.41)	235.71; CI (139.30–332.13)
cyclin D	380.21; CI (361.38–399.05)	305.85; CI (220.65–391.05)
cyclin E	357.13; CI (318.85–395.40)	257.25; CI (116.05–398.45)
$p21^{waf1/cip1}$	334.75; CI (290.05–379.45)	318.33; CI (243.15–393.51)
$p16^{ink4a}$	357.13; CI (318.85–395.40)	239.18; CI (131.22–347.13)
$p27^{kip1}$	359.66; CI (339.36–379.95)	261.94; CI (172.85–351.02)
pRb	362.59; CI (333.28–391.89)	291.57; CI (205.58–377.56)

[1]Mean survival.
[2]Confidence Interval 95%.

Our investigation represents the first comprehensive immunohistological assessment of relevant cell cycle markers including cdc25A, cyclin A, cyclin D, cyclin E, $p16^{ink4a}$, $p21^{waf1/cip1}$, $p27^{kip1}$, and pRb expression in breast cancer tissue and adjacent benign breast tissue of 69 breast cancer patients. According to the St. Gallen 2005 consensus conference (13), we grouped the patients to a low/intermediate and a high risk cohort. High risk patients had a significantly ($p = 0.003$) shorter mean and median survival periods (282.5 weeks; 383.0 weeks, respectively) than low/intermediate risk patients (375.41 weeks; median not reached yet, respectively) in an univariate analysis. Including the above mentioned cell cycle markers in the St. Gallen model of traditional prognostic factors, it was striking to see that in particular cdc25A, cyclin A, cyclin E, $p16^{ink4a}$, and $p27^{kip1}$ influenced survival. Although this influence on survival was statistically not significant in our study, there was an obvious tendency towards a shortened overall survival (Table 3). It is a matter of speculation whether the lack of significance is only due to the small number of samples analyzed. However, whether the metastatic potential in high risk breast cancer patients is dependent on the dysregulation of a single cell cycle marker or of a combined dysregulation of two or more cell cycle markers is not known yet and represents a question to be further investigated.

Defining the individual risk of developing metastases precisely is of enormous importance for the choice of adequate therapy during the early course of the disease because the fifteen-year survival of patients receiving adjuvant therapy in breast cancer has increased from 3% to 10% only (20). Recent data support the idea of mutagenic changes early in the development of breast cancer (21). But, the meaning of genetic changes early in the course of breast cancer in respect to the later development of metastatic disease and survival in low/intermediate risk as well as in high risk patients is still an area of uncertainty. Thus, we had a special interest in this study to find out whether cdks and cdk inhibitors are overexpressed in benign glandular epithelial cells of the breast being adjacent to the breast cancer tissue and whether they influence metastazation and survival. In our investigation only cdc25A, p27kip1, and pRb were found to be positive in the immunohistochemical stain, while all other cell cycle regulators were not. Of interest, 59.4% of our patients overexpressed cdc25A, 63.2% had a low $p27^{kip1}$ expression and 23.2% overexpressed pRb in normal breast tissue adjacent to breast cancer tissue. In the case of $p27^{kip1}$ recent studies used this marker merely as positive control for immunohistochemical staining in benign breast tissue (16, 22). Although we could not detect a significant association between cdc25A, $p27^{kip1}$, and pRb expression and overall survival or disease-free survival in low/intermediate or high risk patients, the fact that these cell cycle regulators are expressed in normal breast tissue adjacent to breast cancer tissue may play an important role in the early phase of breast carcinoma tumorigenesis and in the process of developing metastases, whereby this hypothesis seems to be supported by previously published studies (23–25).

Our results regarding the prognostic value of cdc25A, cyclin A, cyclin E, $p16^{ink4a}$, and $p27^{kip1}$ are in line with others (16, 26-28), but previous studies have also yielded some contradictory results regarding the prognostic value of the different cdks and their inhibitors (15, 29, 30). Actually, it is difficult to compare studies with one another because of the heterogeneity of immunohistochemical techniques, antibodies, and scoring techniques used in the different analyses as has been reviewed by Jacobs et al. (31).

In conclusion, the data of our study demonstrate a shortened overall survival of high risk breast cancer patients defined according to the St. Gallen 2005 consensus conference who additionally overexpress cdc25A, cyclin A, cyclin E, $p16^{ink4a}$, and at a lower level $p27^{kip1}$.

Considering the results presented and discussed, cell cycle markers may have the potential of further refining the risk assessment in breast cancer patients. As a consecutive step confirmation of our results within the scope of a prospective study with a larger patient population and on the basis of standardized laboratory procedures is warranted.

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Coexpression of the Melatonin Receptor 1 and Nestin in Human Breast Cancer Specimens

Olga Rögelsperger, Cem Ekmekcioglu, Walter Jäger, Martin Klimpfinger, Robert Königsberg, Dagmar Krenbek, Franz Sellner, and Theresia Thalhammer

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Coexpression of the melatonin receptor 1 and nestin in human breast cancer specimens

Abstract: Activation of the G-protein-coupled receptor (GPCR) for melatonin (MT1) suppresses breast cancer cell growth in experimental models. To elucidate whether MT1 might play a role in cancer cells positive for the stem cell marker nestin, we assessed paired carcinomatous (Ca) and adjacent noncancerous (NCa) samples from 42 patients with primary breast cancer for MT1 and nestin by double immunofluorescence staining and quantitative image analysis with Tissue-Quest® software. MT1 was located in luminal and myoepithelial cells in milk ducts and in tumor cells in 40/42 and 39/42 of NCa and Ca specimens, respectively, independent of hormone receptor and HER-2 status. Nestin was located together with MT1 in myoepithelial cells in 38 NCa specimens (total n = 42) and in 18 Ca specimens with intact milk ducts. Quantitative evaluation of selected 16 NCa and Ca samples revealed that MT1 levels were higher in invasive Ca sections than in NCa specimens in eight and lower in six cases. Specimens from higher tumor stages (TII/III) with a higher risk of relapse were associated with MT1/nestin co-staining in more than 10% of tumor cells, whereas a lack of co-staining correlated with lower tumor stages. Abundant expression of MT1 and, particularly, coexpression of MT1 with nestin in invading tumor cells in more advanced tumors suggest an important role for this GPCR in the pathogenesis of breast cancer.

O. Rögelsperger[1], C. Ekmekcioglu[2], W. Jäger[3], M. Klimpfinger[4], R. Königsberg[5], D. Krenbek[1], F. Sellner[6] and T. Thalhammer[1]

[1]Department of Pathophysiology and [2]Department of Physiology, Center for Physiology, Pathophysiology and Immunology, Medical University of Vienna, Vienna; [3]Department of Clinical Pharmacy and Diagnostics, University of Vienna, Vienna; [4]Institute of Bacteriology and Pathology, Kaiser-Franz-Josef-Spital, Vienna; [5]CEADDP Applied Cancer Research, Institution for Translational Research Vienna and Ludwig Boltzmann Institute for Applied Cancer Research, Vienna; [6]Clinic for Surgery, Kaiser-Franz-Josef-Spital, Vienna, Austria

Key words: breast cancer, MT1–nestin coexpression, immunofluorescence, quantification, Tissue-Quest®

Address reprint requests to Cem Ekmekcioglu, MD, Department of Physiology, Center for Physiology, Pathophysiology and Immunology, Medical University of Vienna, Schwarzspanierstrasse 17, A-1090 Vienna, Austria.
E-mail: cem.ekmekcioglu@meduniwien.ac.at

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Introduction

Melatonin acts via receptor-independent antioxidative [1, 2] and receptor-mediated mechanisms [3]. Previous studies showed that melatonin inhibits proliferation of breast cancer cell lines and prevents progression of malignant breast tumors in animal models [4]. These anticancer effects of melatonin are thought to be mediated at least in part by binding and activation of its high-affinity G-protein-coupled receptor (GPCR), the melatonin receptor (MT) 1 [5, 6]. This receptor has been detected in various human tissues [7]. Many studies used the estrogen-sensitive breast cancer cell line MCF-7 overexpressing MT1, and showed that MT1 activation suppresses estrogen receptor (ER)-induced transcription of genes important for cell proliferation and tumor progression, resulting in the inhibition of breast cancer growth [8, 9]. Therefore, MT1 activation induces a strong antiproliferative effect in ER-expressing breast tumors sensitive to circulating estrogens [10]. This is important for a large number of breast cancers, which express ER and that are responsive to anti-hormonal therapy.

The success of this therapy, however, is often temporary as tumors escape their estrogen responsiveness and growth progresses [11]. With respect to this escape from estrogen dependency, it is interesting that in an immunohistochemical study in patients with breast cancer, the expression of MT1 was found to be higher in less differentiated breast tumors, independent of the expression of ER and also of the progesterone receptor (PR) [12]. Therefore, MT1 expression seems to be important for additional mechanisms, which could influence tumor growth independent of

estrogen signaling. For example, in breast cancer cell lines, MT1 signaling was shown to interfere with the transcriptional activity of the glucocorticoid receptor and the retinoid receptor involving different G-protein signaling pathways [13]. Moreover, the inhibitory effect of melatonin on the uptake of tumor growth promoting fatty acids and the suppression of fatty acid metabolism could be important in these tumors [14].

In line with these findings, other studies showed that the activation of MT1 by melatonin is indeed associated with an induction of cell differentiation of neuronal as well as non-neuronal cells and also of tumor cells. For example, it induces neurite outgrowths in neuroblastoma cells and causes neuroendocrine differentiation of prostate cancer cells [15, 16]. In MT1-transfected Chinese hamster ovary (CHO) cells, receptor activation by melatonin provokes changes in cellular properties, e.g. induction of filamentous structures, dependent on activation of the MEK1/2 ERK1/2 signaling pathway [17].

While these studies very well explain the anticancer effects of MT1 activated by melatonin, the high MT1 expression levels in cancer tissue, as found in the breast cancer study of Dillon et al. [12] are still not easy to explain and, therefore, another function of MT1 seems likely. Like other GPCRs, MT1 might have the ability to become activated independent of its ligand melatonin, thereby changing the fate of normal and transformed cells in a still unknown way [18, 19]. This might be of particular significance for tumor induction and progression and could occur in mature cells as well as in their progenitor/stem cells, as cells with a capacity of unlimited renewal, differentiation and motility [20].

Similar to normal stem cells, also in cancer, a small population of cells, termed cancer stem cells (CSC), possess the capability for self-renewal, asymmetric cell division and indefinite proliferative capacity. These tumor-initiating CSCs have been identified in a variety of hematological and solid malignancies, and were also found in breast cancer [21]. CSCs share various characteristics with normal stem cells, but they also contain a unique and disease-specific signature of proteins [22]. For example, nestin, an intermediate filament protein, was originally identified as a marker of neuroepithelial stem/progenitor cells in the brain, but was also later found in a cell population with CSC characteristics in many tumors, e.g. breast cancer [23–25]. It is assumed that nestin-expressing stem cells initiate a population of dedifferentiated cells capable of unlimited growth giving rise to highly undifferentiated tumors. This model for malignant transformation in the breast is supported by the finding that high nestin levels were associated with aggressive, poorly differentiated basal-like breast cancer [24]. Moreover, due to their nestin expression, endothelial cells in newly formed capillaries are thought to have developed from nestin-positive precursor cells, and their presence is associated with a highly aggressive phenotype of cancer [25].

Importantly, expression of nestin together with MT1 was found in neural stem cells [26], but a possible association of nestin together with MT1 has not yet been evaluated in breast cancer cells. Therefore, the present study was carried out to elucidate whether MT1 expression in different cells might correspond to that of nestin. We further investigated whether this possible coexpression might be associated with particular features of the tumors (grading, tumor stage and risk of relapse). Investigations were carried out in the cancerous (Ca) and the adjacent noncancerous (NCa) parts of breast tissue specimens from the same patient using a novel quantitative image analysis system for the evaluation of the immunofluorescence data.

Materials and methods

Patients

Forty-two patients with primary, invasive breast carcinoma, diagnosed and operated between 1998 and 2003 at the Kaiser-Franz-Josef Hospital, Vienna, were enrolled in the study. Written informed consent was obtained from all patients, and the study was approved by the ethics committee of the institution.

The mean age of patients at primary diagnosis was 56 ± 10 yr (range 29–76). Patients were classified as described by Königsberg et al. [27]. Staging was carried out at the primary diagnosis according to the guidelines of the International Union against Cancer (UICC). The observation time after the date of primary diagnosis to the date of last appointment or disease-associated death was 67 ± 19 months, ranging from 11 to 96 months. Based on data on patient age, tumor size, presence of tumor cells in local lymph nodes, tumor differentiation grade, HER2 status and peritumoral vascular invasion, patients were assigned to be at low, intermediate or high risk of local or distant relapse [28]. From eight patients with distant metastases, lungs were affected in 87.5%, liver in 62.5%, bone in 37.5% and brain in 25.0% of patients. Seven of 42 (16.7%) patients died from metastatic disease within the observation time.

All tissue specimens were obtained from patients prior to chemotherapy by surgical excision immediately after primary diagnosis and, in our study, parts not required for further routine diagnosis were used. All samples were routinely subjected to pathological examination. Twenty-nine patients had invasive ductal carcinoma, six invasive lobular carcinoma and seven patients had tumors with other histological features (e.g. apocrine, medullary or mucinous carcinoma). Grading was carried out according to Bloom and Richardson, with 4 G1, 21 G2 and 17 G3 tumors. ER (27 positive), PR (16 positive) and HER2 (19 positive) expression was routinely assessed using immunohistochemistry at the Institute of Bacteriology and Pathology, Kaiser-Franz-Josef-Hospital, Vienna. The evaluation of ER and PR status was carried out according to the method of Remmele and Stegner [29], and HER2 staining were regarded positive if >10% of cells showed distinct and complete membrane staining of HER2.

Antibodies

The rabbit polyclonal antibody against the human MT1 was purchased from Abcam, Cambridge, UK. A mouse monoclonal antibody (mAB) against nestin (clone 10C2)

was obtained from Millipore (Temecula, CA, USA). Both primary antibodies were applied at a concentration of 2.5 μg/mL. The mouse mAB against CD10/CALLA (clone 56C6) and the mAB against α-SMA (clone 1A4) were both obtained from Labvision/Neomarkers (Fremont, CA, USA), and were applied at concentrations of 1:30 tissue culture supernatant and 0.25 μg/mL respectively.

As secondary antibodies, we applied Alexa Fluor® 488 (green) and 568 (red) labeled goat anti-rabbit (488), and goat anti-mouse (568) antibodies at concentrations of 2 μg/mL, which were obtained from Molecular Probes (Eugene, OR, USA). For immunohistochemical detection of MT1, a goat anti-rabbit/mouse antibody conjugated to horseradish peroxidase labeled dextran (Envision®) was used as a secondary antibody and purchased from Dako Company (Glostrup, Denmark).

For negative control, the following immunoglobulins (Ig) were applied: rabbit anti-mouse IgG (Pierce, Rockford, IL, USA) and mouse myeloma IgG1 (Zymed Laboratories, South San Francisco, CA, USA).

Immunohistochemical detection of MT1

After surgical excision of the breast carcinoma and the surrounding nonmalignant resection margin, both tissues were routinely fixed in formalin (4%), dehydrated and embedded in paraffin. From tissue blocks, 1-μm sections were prepared, mounted on adhesive slides and dried for at least 1 hr at 65°C. Thereafter, sections were deparaffinized in xylene, and the endogenous enzyme activity was blocked with 3% hydrogen peroxide in methanol for 10 min at room temperature (RT). The sections were then processed through a graded series of alcohols and rehydrated in phosphate-buffered saline (PBS). After permeabilization in 0.1% Tween-20 in PBS for 10 min at RT, antigen retrieval was performed using the microwave technique in 10 mM citrate buffer (pH 6.0) for 10 min, followed by blocking with 10% bovine serum albumin (BSA) in PBS for 30 min. Thereafter, the sections were incubated with the MT1 antibody overnight at 4°C in a humidified chamber. After washing three times in PBS, the dextran polymer peroxidase Envision System was applied for 30 min at RT. The antigen was then visualized using the 3′,3-diaminobenzidine chromogen for 4 min. Cell nuclei were counterstained with hematoxylin (ChemMate®; Dako), rinsed with distilled water and mounted in Mowiol (Sigma-Aldrich GmbH, Steinheim, Germany).

Immunofluorescence staining

Antigen retrieval and blocking were performed as described in the immunohistochemistry experiments. For MT1 staining, the sections were incubated with the MT1 antibody overnight at 4°C. This step was followed by washing three times in PBS. Alexa Fluor® 488-conjugated goat anti-rabbit IgG was applied as secondary antibody for 1 hr at RT.

For MT1–nestin double staining, initially, staining was performed with an anti-nestin mAB for 1 hr (RT). This step was followed by washing in PBS 3 × 5 min, and the staining was completed by incubation with Alexa Fluor® 568 goat anti-mouse IgG for 1 hr at RT. Thereafter, slides were washed in PBS and blocked again with 10% BSA in PBS for 30 min before staining with the second primary antibody against MT1 (overnight, 4°C) was performed. As a secondary antibody, Alexa Fluor® 488-conjugated goat anti-rabbit IgG (1 hr at RT) was used.

For CD10 single staining, heat-induced antigen retrieval in 1 mM EDTA (pH 8.0) was performed for 10 min. No antigen demasking was necessary for α-SMA detection. Both antibodies were applied overnight at 4°C in a humidified chamber. After washing in PBS for 3 × 5 min, sections were incubated with Alexa Fluor® 568 goat anti-mouse IgG for 1 hr at RT.

For negative controls, the primary antibodies were replaced by the isotype-matched control IgG. Cell nuclei were counterstained with 0.5 μg/mL bisbenzimide in PBS (Hoechst 33342; Sigma, Munich, Germany). After completing the immunofluorescence staining, slides were rinsed with distilled water, dried and mounted in H-1000 Vectashield medium (Vector Laboratories Inc., Burlingame, CA, USA).

Microscopy and image collection

Sections were viewed in an Axioplan 2 microscope (Carl Zeiss, Jena, Germany). For each tissue section, monochrome images of four representative fields were recorded from each channel with an AxioCam HRc2 Color CCD digital camera at 10× and 40× magnification respectively. To minimize background signals, the exposure times for the MT1 (and other antigens) antibody staining were evaluated for a certain magnification and kept constant between the samples. Background subtraction was uniformly carried out with the Axiovision 4.6 software (Carl Zeiss Vision GmbH, Aalen, Germany).

Quantification of MT1 and nestin immunostaining

All carcinoma and corresponding noncarcinoma sections showing distinct MT1 staining where a clear localization of MT1 could be recognized were further analyzed for the number of cells with MT1 and nestin immunostaining. Of each tissue section, four representative images (40× magnification) were analyzed using the Tissue-Quest® 2.2 software (TissueGnostics GmbH, Vienna, Austria). Single cells were identified by their nuclei (blue bisbenzimide staining). This identification mask was then used to measure gray values in the two corresponding channels (MT1 and nestin) of each object in all images [30].

For the quantification of MT1- and nestin-containing cells, their fluorescence intensity signals were plotted against the bisbenzimide signal, as a parameter for the cell number, in scattergrams. Each scattergram represents average values calculated from analysis of all four images of one entire tissue section. Based on the degree of fluorescence intensity in the negative controls (control IgG), appropriate threshold values for positive immunofluorescence staining were defined. Because isotype controls were always less than 10%, the threshold for considering the samples positive for MT1 or nestin staining was set at 10%. MT1 staining levels were considered to be different

between cancer and noncancer tissues, when the difference was ≥10%.

Statistical analysis

All calculations were performed with the SPSS 14.0 statistical software package (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± standard deviation, and were compared with the two-sided paired or unpaired *t*-test, if normally distributed. Otherwise, the two-sided Mann–Whitney *U*-test was used. Differences between two groups of categorical variables were tested with the two-sided Fisher's exact test. A value of $P \leq 0.05$ was considered statistically significant.

Results

Overall, in paired samples from 37/42 patients green staining for MT1 immunoreactivity was observed in the Ca and adjacent NCa specimens (Fig. 1). In NCa sections, MT1 immunoreactivity is located in myoepithelial and luminal epithelial cells of the milk ducts. Occasionally, green MT1-stained stromal cells are visible of normal tissue (Fig. 1A,B). The inset in Fig. 1A further shows that MT1 immunoreactivity is mainly located in the cell membrane of ductal epithelial cells in this specimen. This was only seen in 15/42 NCa specimens, while in 10/42 of NCa specimens, in addition to cell membranes, cytosolic compartments were also stained for MT1. In the remaining 15/42 MT1-positive samples, the subcellular localization of MT1 was diffuse and not clearly attributable to the membrane or the cytosol. An analogous staining pattern for MT1 was also observed in immunohistochemistry experiments (Fig. 1C).

In contrast to the more frequent membrane, MT1 localization in the NCa specimens, MT1 immunolocalization in the cytosol was seen in the majority of all Ca specimens (21/42), while preferential cell membrane staining was seen in 10/42 Ca samples. In the remaining eight samples, the localization of MT1 was not clearly attributable to either group. However, comparison of the subcellular localization of MT1 in Ca and paired NCa sections did not reveal significant differences between the two groups ($P = 0.74$). MT1 immunofluorescence staining patterns of Ca sections from a highly undifferentiated (G3) invasive ductal tumor are shown in Fig. 1D,E. Fig. 1F represents an immunohistochemical staining for MT1 in a better differentiated ductal carcinoma (G2). As demonstrated in the inset of Fig. 1D, MT1 immunoreactivity is

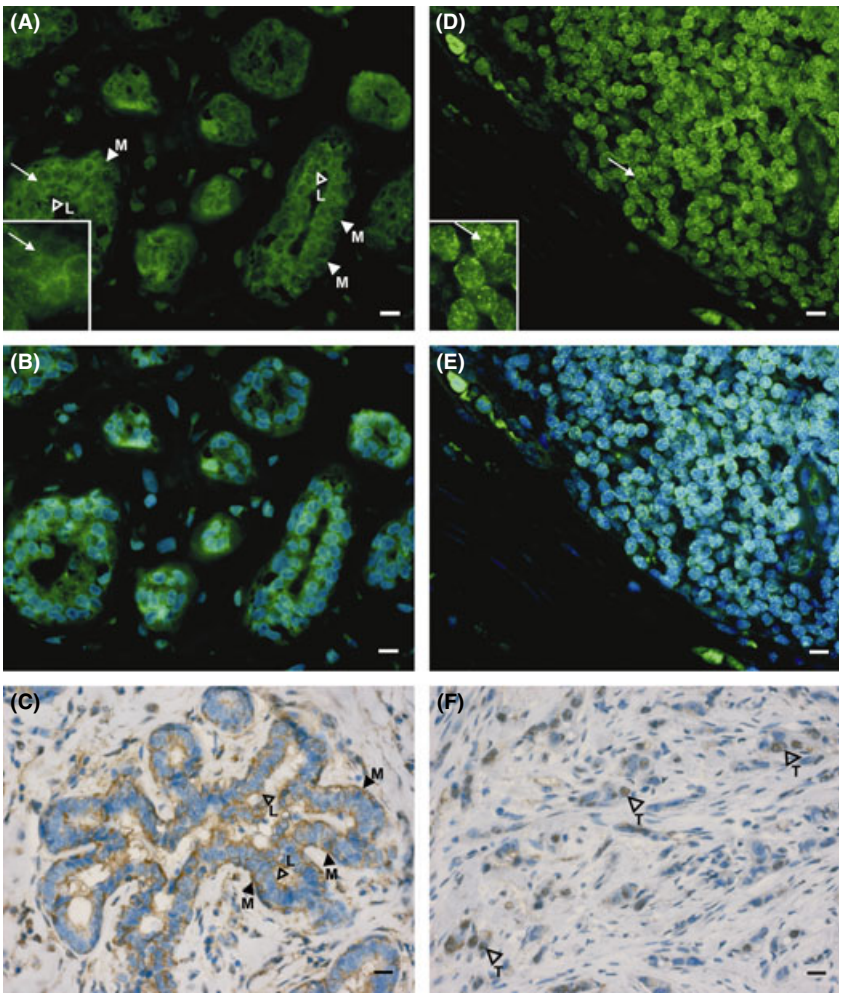

Fig. 1. Expression of MT1 in noncancerous and cancerous breast tissue. Paraffin-embedded sections from breast tissue were stained for MT1 by immunofluorescence (A,B,D,E) and immunohistochemistry (C,F). MT1 staining of epithelial cells in milk ducts is seen in noncancerous tissue (A–C). In milk ducts, outer myoepithelial cells (M) and inner luminal cells (L) show green MT1 fluorescence (A,B) and brown staining of MT1 with DAB (C). As shown in the inset (arrow), MT1 staining is mainly in the membrane. MT1 staining of carcinoma cells (T, arrowheads in image F) of invasive ductal tumors of G2 (F; patient 12) and G3 (D,E; patient 17). The area shown at higher magnification in the inset is marked by an arrow. Green spots in carcinoma cells suggest MT1 localization in cytoplasmic compartments. Note that the images (B) and (E) correspond to (A) and (D), but are additionally counterstained with the DNA intercalating dye bisbenzimide. In (C) and (F), nuclei were counterstained with hematoxylin. Inset bars represent 10 μm. Number of samples correspond to that in Table 1 and Fig. 3 (panel B).

spotted in cytoplasmic compartments in ductal epithelial cells in this Ca specimen.

MT1 immunoreactivity in cell membranes and in cytoplasmic compartments, or both, was further verified by confocal immunofluorescence laser scanning microscopy (data not shown). As nestin was previously described to be expressed in breast tumors in myoepithelial and cancer cells with stem cell properties [23, 24], we investigated nestin immunolocalization with respect to that of MT1 to: (i) confirm myoepithelial localization of MT1 and (ii) identify cancer cell subpopulations positive for nestin and MT1.

In the immunofluorescence analysis, overall positive cytoplasmic nestin staining was observed in 38/42 of NCa, where it was located in myoepithelial cells in milk ducts and lobules. From 31 nestin-positive Ca specimens, in nine nestin was seen only in myoepithelial cells of milk ducts, in another nine in myoepithelial cells and infiltrating cells and in the remaining 13 in infiltrating tumor cells only. In the latter, nestin was observed in tumor cell clusters and vascular-like structures (Fig 2A and B). Double staining for MT1 and nestin is shown in Fig. 3A(a–c) in NCa and Ca sections from a patient with an invasive ductal carcinoma (G2). In parts of this tumor, intact milk ducts are still present. MT1–nestin coexpression is found in myoepithelial cells of the milk ducts in the NCa and Ca sections (Fig. 3Aa,b), while in image c, overlap of MT1 and nestin staining is found in only a small fraction of tumor cells.

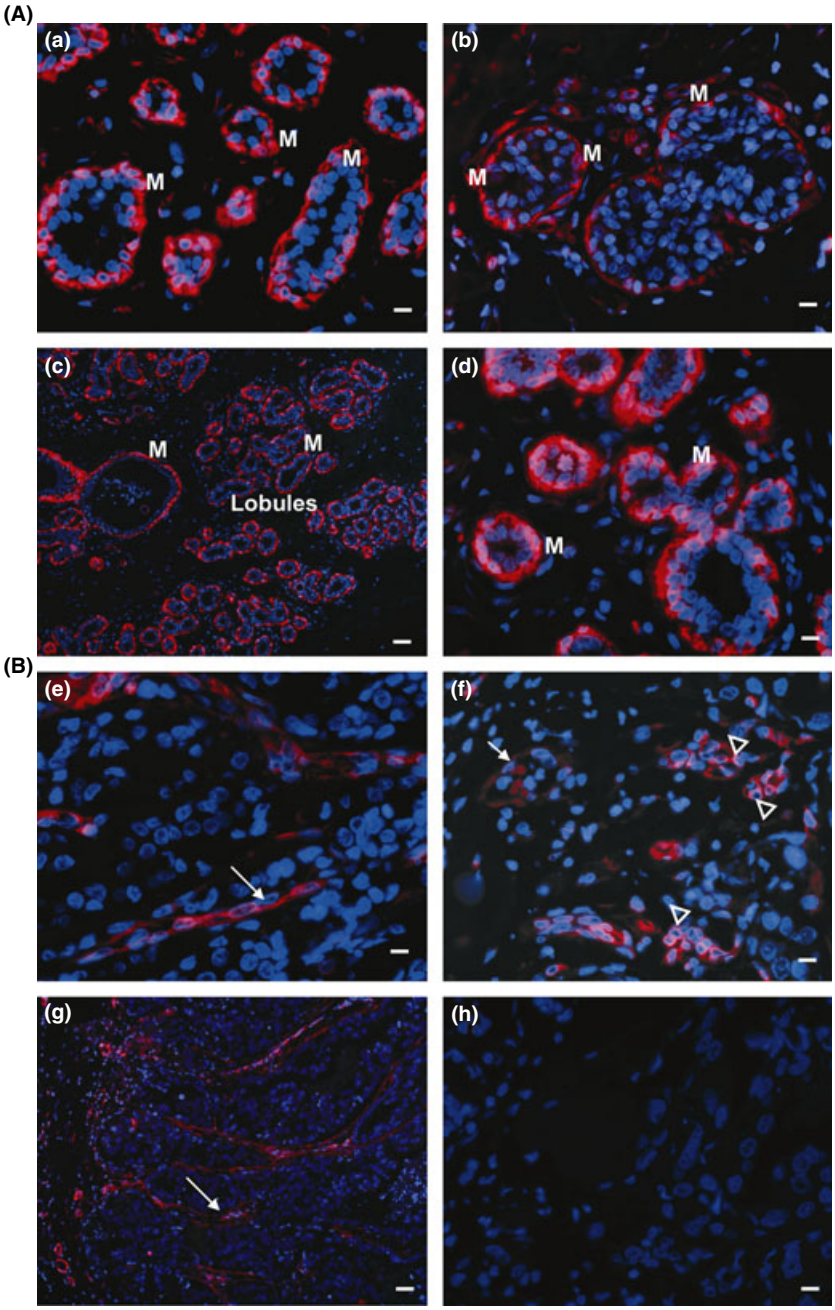

Fig. 2. Immunofluorescence staining of nestin, α-SMA and CD10 in malignant and nonmalignant breast tissue. (A): Red nestin staining of milk ducts in malignant (b) and nonmalignant (a) tissue. Image (b) shows the intraductal component of an invasive ductal carcinoma, G2, from patient 3. (c) shows red α-SMA and (d) red CD10 staining of milk ducts and lobules in nonmalignant tissue. (B) These serial sections show an apocrine tumor (G2), obtained from patient 2. Red nestin staining in the invasive part of the tumor, without milk duct structures (e,f). Note, that long stretched structures of nestin staining (e, indicated by arrows) resemble the pattern of α-SMA staining in picture (g). In (f), nestin-positive cells are forming clusters (indicated by arrowheads), which are negative for the myoepithelial marker CD10 (h). The arrow indicates nestin expression in endothelial cells of a blood vessel. (g) shows red α-SMA staining in the invasive part of the tumor, without milk duct structures. (h) No staining for CD10 is observed in this tumor. Cell nuclei were detected by staining of the DNA with bisbenzimide (blue). Inset bars represent 10 μm for nestin (a,b,e,f) and CD10 (d,h), and 40 μm for α-SMA (c,g) staining pictures.

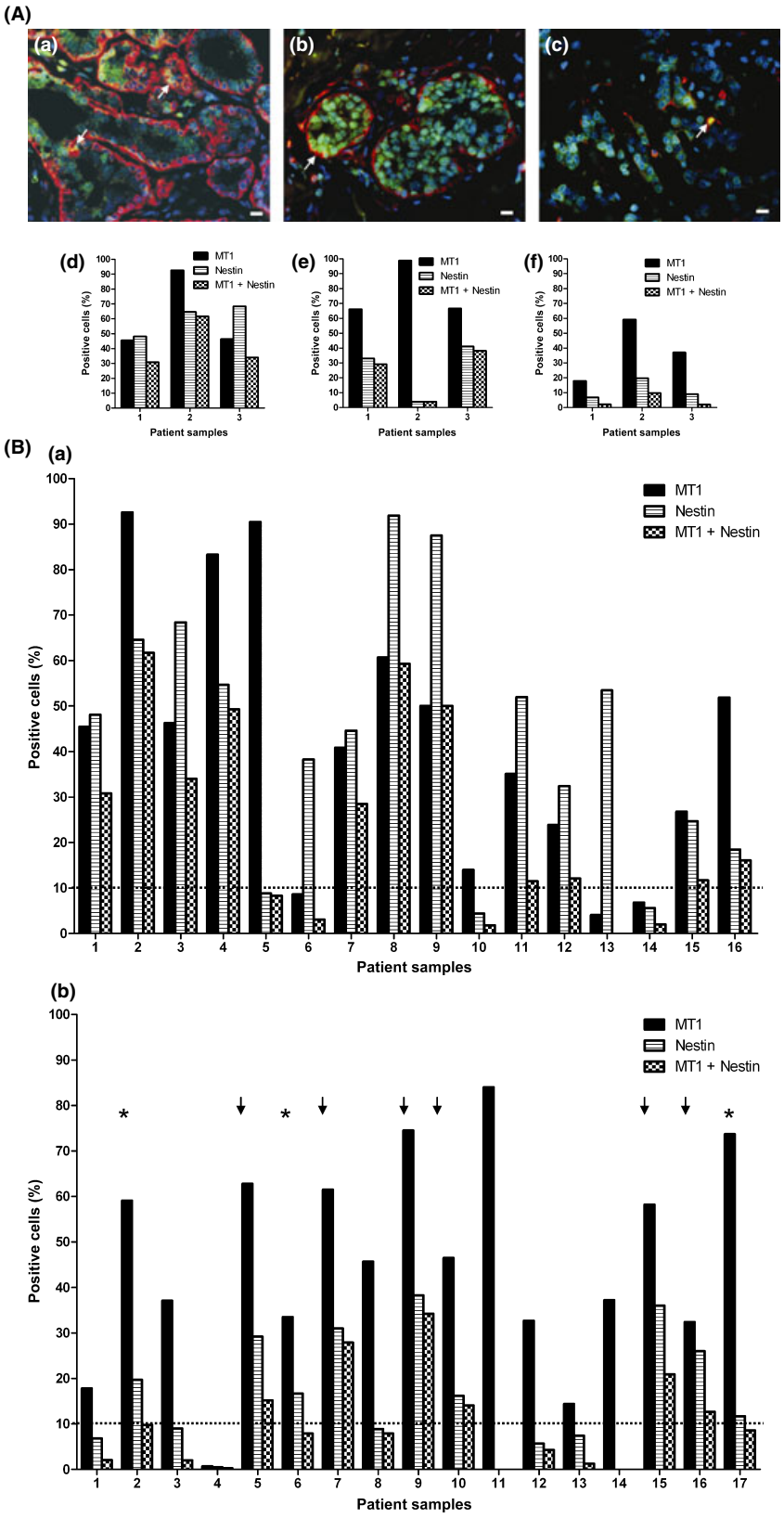

Fig. 3. MT1- (green) and nestin (red)-co-staining in the noncancerous (a) and cancerous parts (b,c) from breast cancer specimens and its quantitative evaluation. (A) (a–c) Coexpression is seen in milk ducts in the noncancerous (a) and cancerous (b) parts and in cells in the invasive component (c) from patient 3 (see B) with a G2 invasive ductal tumor (c). In the merged picture of green MT1 and red nestin staining, orange to yellow staining (arrows) indicates overlay of MT1 and nestin fluorescence. Inset bars: 10 μm. (d–f) Quantitative evaluation of MT1-, nestin- and MT1–nestin-coexpressing cells. Data were obtained from Tissue-Quest® 2.2 image analysis of specimens from patient 3 (shown above), and from two additional patients. Noncancerous (d) and cancerous (e) milk ducts and invasive component (f). Note, that only patient 2 had $>10\%$ of MT1 and nestin double-positive cells in the invasive part of the tumor respectively. (B): Number of MT1-, nestin- and MT1–nestin-coexpressing cells in specimens from noncancer (a) and invasive cancer (b) sections from breast tumors from 17 patients. The percentage of MT1- (black bars), nestin- (shaded bars) and MT1–nestin (checkered bars) coexpressing cells was calculated from data in the scattergrams, where cells in the upper right quadrants were considered MT1 positive. In panel (b), the stars and the arrows indicate patients with high numbers of MT1- and nestin-expressing cells, showing $<10\%$ (stars) and $>10\%$ (arrows) of MT1–nestin-coexpressing cells respectively. *For patient 17, NCa tissue was not analyzable.

To estimate the percentage of cells in the Ca and NCa samples, we applied quantitative Tissue Quest analysis. For this analysis, paired Ca and NCa samples were chosen in which MT1 membranous and/or cytoplasmic staining was pronounced in a uniform pattern in the whole specimen. This was the case for 17 patients, but for patient 17, only

Table 1. Characteristics of patients, whose specimens were quantitatively assessed for the number of MT1 and nestin-positive cells

Patient no.	Age[a] (yr)	Stage	Histology	Grade	ER	PR	HER-2
1	63	I	Invasive ductal	1	+	+	–
2	47	I	Apocrine	2	–	–	+
3	55	I	Invasive ductal	2	–	–	+
4	68	IIA	Invasive ductal	2	+	–	–
5	53	IIA	Invasive ductal	3	–	–	–
6	42	IIB	Invasive ductal	2	–	+	+
7	68	IIA	Invasive ductal	2	+	+	–
8	75	IIA	Invasive ductal	3	–	–	+
9	29	IIA	Invasive ductal	3	+	+	+
10	71	IIIA	Invasive ductal	2	+	+	+
11	66	IIA	Invasive ductal	2	+	–	–
12	49	IIA	Invasive ductal	2	–	+	+
13	57	IIA	Medullary	3	–	–	+
14	53	IIA	Invasive ductal	3	–	–	+
15	64	IIB	invasive lobular	2	+	+	–
16	44	IIIC	Invasive lobular	3	–	–	+
17	44	I	Invasive ductal	3	–	–	+

Patient numbers correspond to Fig. 3B.
[a]Age at primary diagnosis.

the Ca tissue was available. Clinical data of these 17 patients are given in Table 1. We found that: i) 8/16 tumors had more MT1-positive cells (>10%) in the Ca than in adjacent NCa section; ii) in 6/16 tumors, the number of MT1-positive cells was higher (>10%) in the NCa than in corresponding Ca section; and that iii) the remaining two patients showed no difference (<10%) between the NCa and Ca sections in the amount of cells positive for MT1.

In Fig. 4A, images (a–c) and the corresponding scatter plots (d–f) are given for the quantification of MT1-expressing cells in NCa and surrounding invasive Ca specimens from a representative patient. In Fig. 4B, data on the analysis of MT1-expressing cells from invasive Ca and corresponding NCa sections from 16 patients (Table 1) are depicted.

We found no significant relationship between the amount of MT1-expressing cells in the invasive Ca and ER, PR or HER2 status, the menopausal status and the tumor differentiation grade. Moreover, quantification of cells expressing nestin was carried out in the Ca and NCa specimens from the 17 patients (Table 1). Nestin was found in 13/16 NCa specimens, and in 9/17 Ca specimens.

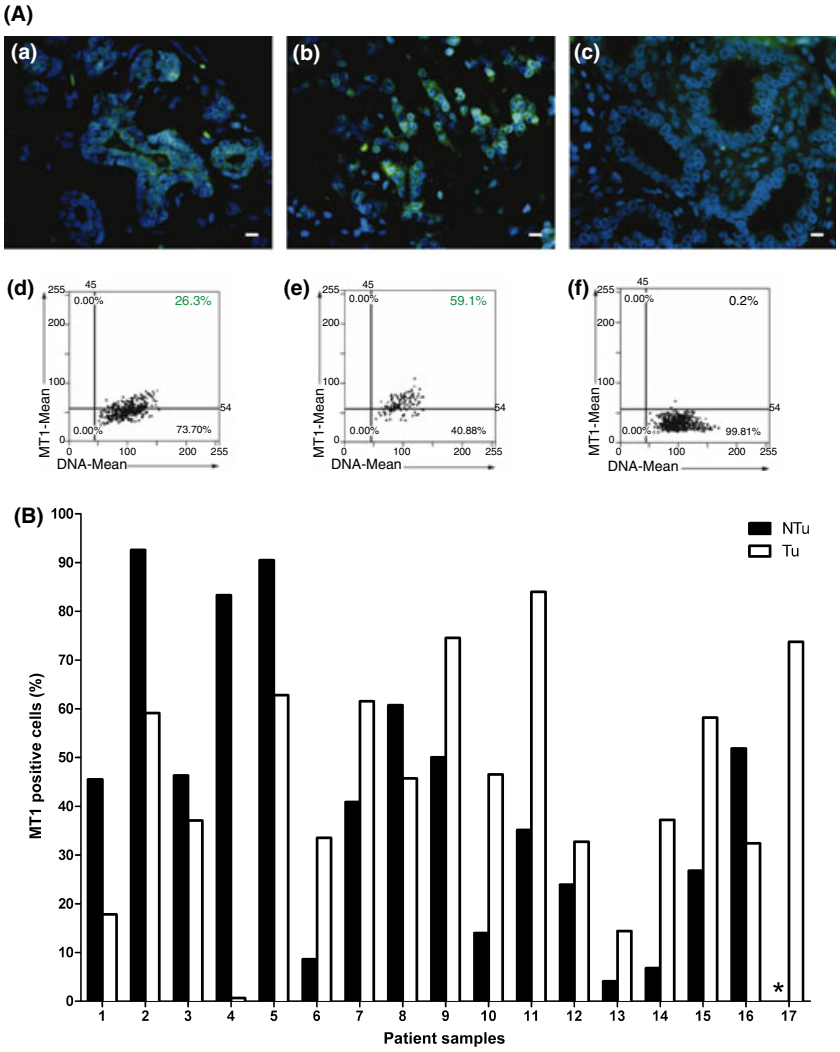

Fig. 4. Quantitative evaluation of MT1 levels in cancerous and adjacent noncancerous breast specimens. (A) Immunofluorescence staining of MT1 (green) in nonmalignant (a) and malignant tissue (b) from patient 15 with invasive lobular breast carcinoma (G2). Nuclei (blue) were counterstained with bisbenzimide and were used for cell recognition (DNA mean) in the image analysis. Their number is given in the dot blots (d–f) which correspond to the immunofluorescence pictures (a–c). Cells were analyzed for DNA content (*x*) and MT1 fluorescence (*y*). The upper right quadrant in the dot blot shows the number of MT1 stained cells in one specimen. Detection thresholds are indicated by black lines, which have been set after image analysis of the negative control (noncancerous breast tissue, c,f), in which unspecific control IgG were used instead of the MT1 antibody. Inset bars represent 10 μm. (B) Number of MT1-expressing cells in specimens from noncancer (black bars) and invasive tumor (gray bars) sections from breast tumors from 17 patients. The percentage of MT1-expressing cells was calculated from data in the scattergrams, where cells in the upper right quadrants were considered MT1 positive. *For patient 17, NCa tissue was not analyzable.

Remarkably, these nine patients also had high MT1 staining levels (panel b in Fig. 3B). Therefore, in the next step we investigated whether MT1 might be expressed together with nestin in the same cell.

MT1–nestin double-positive myoepithelial cells were found in 11/16 NCa samples (Fig. 3B image a). As shown in Fig. 3B(b), remarkable MT1–nestin coexpression was found in 6/17 Ca sections lacking intact milk ducts. Representative images of MT1-, nestin- and MT1–nestin overlay staining in invasive parts of Ca from two patients with and without MT1–nestin coexpression are shown in Fig. 5A and B respectively.

In Table 2 we show that MT1 and nestin co-localization is found in six patients being in a more advanced tumor stage (IIA–IIIC). By contrast, patients without remarkable MT1–nestin colocalization were at a low tumor stage (2/3 in stage I). However, in this explorative study, differences in tumor stage between the two groups did not reach significance ($P = 0.083$). In addition, interestingly, 4/6 patients with MT1–nestin coexpression had ER and PR double-positive tumors (two with ER+/PR+/HER2+; and two with ER+/PR+/HER2−), whereas 2/3 patients without remarkable coexpression showed HER2-like phenotype (ER−/PR−/HER2+).

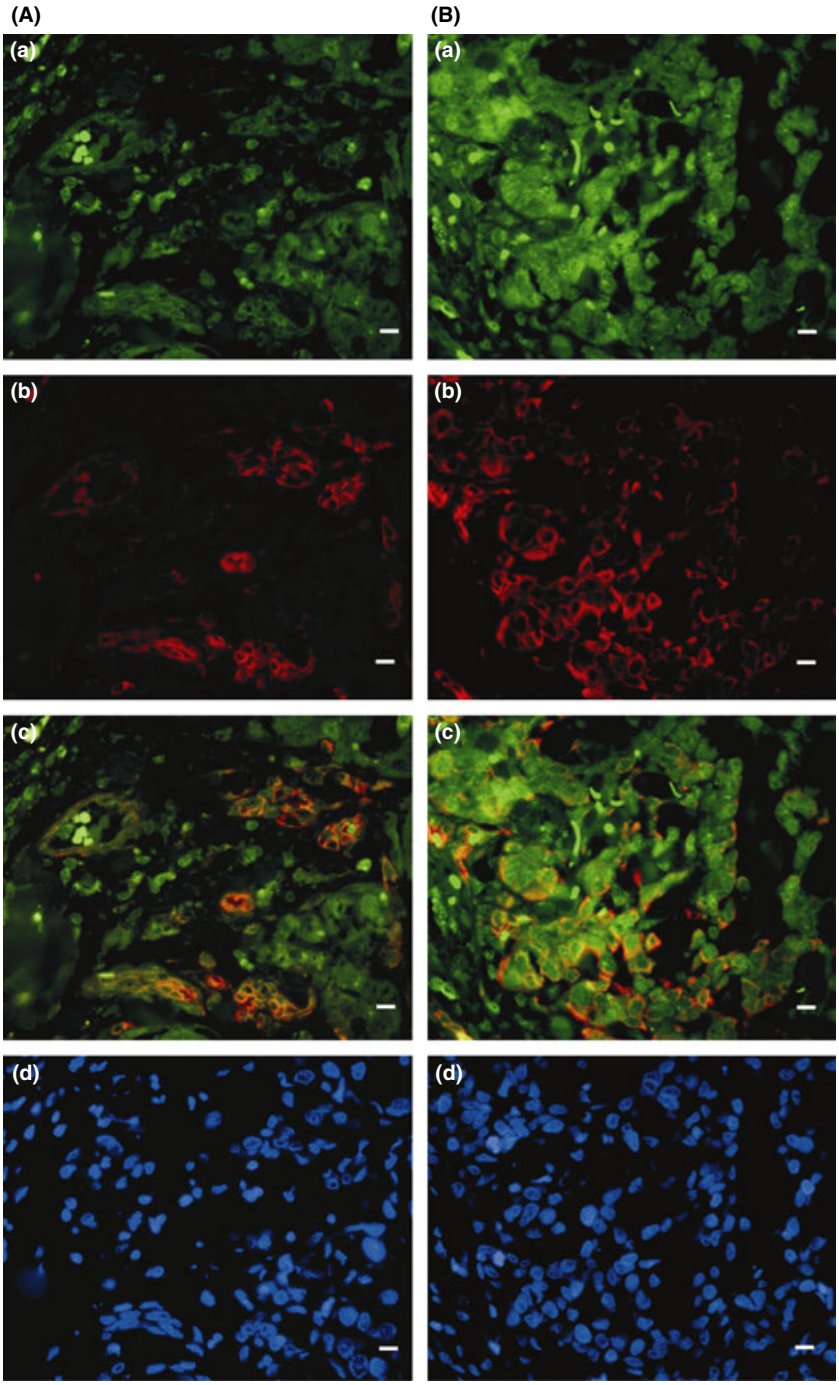

Fig. 5. MT1 and nestin coexpression in invasive tumors containing >10% MT1- and nestin-expressing, CD10-negative cells. Immunofluorescence staining for MT1 (a), nestin (b) and the merged pictures (c) are shown in specimens from a G2 apocrine carcinoma (patient 2, A), and from an invasive ductal G3 carcinoma (patient 5, B). MT1 expression in nestin-positive/CD10-negative cells is seen in <10% in Ca sections from patient 2 (A) and >10% in patient 5 (B). In (d), blue stained cell nuclei in the apocrine (A) and the invasive ductal (B) carcinoma are shown. Inset bars: 10 μm.

Table 2. Characteristics of breast cancer patients without and with MT1 and nestin coexpressing cells in the tumor stroma

	MT1–nestin coexpression	
	Absent (n = 3) Patient no. 2, 6, 17	Present (n = 6) Patient no. 5, 7, 9, 10, 15, 16
Tumor grade		
G1	0	0
G2	2	3
G3	1	3
Stage		
I	2	0
II	1	4
III	0	2
Risk of relapse[a]		
Low	0	0
Intermediate	3	4
High	0	2
Relapse		
No	1	5
Local[b]	2	0
Distant[c]	0	1
Death		
Alive	3	5
Dead	0	1
Age, mean ± SD (yr)	44.6 ± 2.5	54.7 ± 15.9

[a]Risk categories were defined as described in the Materials and methods section.
[b]Local relapse within 49 months after primary diagnosis.
[c]Distant metastases in the lung, brain and bone within 8 months after primary diagnosis. This patient (16, Table 1, Fig. 3B) died from disease 11 months after primary diagnosis.

Discussion

In this study, we showed that MT1 and nestin immunoreactivity was detectable in a great majority of Ca and adjacent NCa specimens from 42 patients with breast cancer. MT1 staining was detected in luminal and myoepithelial cells in milk ducts of NCa and also in differentiated tumor areas, giving a considerable overlap with the nestin staining. In invasive tumor parts, MT1 was present in single tumor cells and cell clusters invading the stroma as well as in perivascular cells, which were also positive for nestin. The striking colocalization of MT1 with the intermediate filament protein nestin raises the question about the role of MT1 in nestin-expressing myoepithelial and also in tumor cells in breast tissue.

Mammary gland structure constantly changes in size, shape and function from the time of puberty to menopause, and it is well known that myoepithelial cells play a key role in these processes [31]. Moreover, the loss of or change in myoepithelial cell function is a key step in the transition from the *in situ* (i.e. tumor limited to the inside of the milk duct) to invasive breast cancer, as, under normal or even premalignant conditions, the myoepithelial cells are forming a structural barrier between the luminal epithelial cells and the surrounding stroma, thus preventing cell invasion [31, 32]. Additionally, myoepithelial cells have been shown to inhibit growth and invasion of breast cancer cells through secretion of paracrine factors, like extracellular matrix proteins and protease inhibitors [31]. These considerations support our findings that MT1 is expressed in some, but not all, nestin-positive myoepithelial cells in NCa and Ca milk ducts. Moreover, the presence of MT1 in luminal, nestin-negative cells, might be important, as the majority of breast cancers are developing from these cells.

We found MT1–nestin coexpression also in carcinoma cells in the invasive Ca parts. Both, luminal and myoepithelial cells are thought to be derived from a common precursor/stem cell, and, interestingly, in a recent experiment it was shown that neural stem cells can settle in a new microenvironment and can adopt the function of similarly endowed mammary progenitors [33]. This could also apply for human breast tumors. Therefore, it is interesting to mention that the coexpression of MT and nestin had recently been reported in a mouse neural stem cell line [26].

Cancer development and progression is characterized by dynamic changes in the expression and function of protein kinases which regulate mitogenesis, motility, invasion, cell survival and angiogenesis [34]. Various alterations in the expression or function of protein kinases or their associated signaling pathways can lead to malignant transformation of cells in the breast [35]. For example, overexpression of several receptor tyrosine kinases and deregulation of the phosphoinositide-3-kinase (PI3K) pathway were found to contribute to the development of breast cancer [36, 37]. This consideration is important, as MT1 activation by melatonin was found to regulate a number of different signaling pathways involving the second messengers cAMP, diacylglycerol, inositol triphosphate (IP3), arachidonic acid and intracellular Ca^{2+} ($[Ca^{2+}]_i$) [38, 39]. However, if melatonin was used for MT1 activation in experimental studies, rather an inhibitory effect on pathways, like the PI3K pathway, was observed, as shown by a recent study in MCF-7 breast cancer cells, where MT1 activation by melatonin, via coupling to G(αq), inhibited phospholipid hydrolysis and the induction of IP3 production [6]. Moreover, an enhancement of the retinoid-induced RARα transcriptional activity is achieved via G(αq) coupling.

Other oncostatic properties of melatonin most likely to be conferred via binding to MT1 are related to its interaction with estrogens and the ER [40]. For example, via epigenetic processes, melatonin downregulates the expression of genes responsible for the local synthesis or activation of estrogens, e.g. aromatase. Melatonin further inhibits telomerase activity and cyclin D1 expression induced by estrogens, thereby modulating the cell cycle [5]. Although these mechanisms could contribute to potential beneficial effects of melatonin on tumor progression, as shown in animal models [10, 14], they are related to the estrogen responsiveness of the cells, i.e. requiring a functional ER. Therefore, the high levels of MT1 found in our study also in ER-negative Ca specimens, are difficult to explain, but tumor suppressive effects of melatonin via MT1 in hormone-independent tumors have also been described in other studies, like in hormone refractory prostate cancer [41]. These findings suggest additional hormone-independent functions of MT1.

Both abundant and aberrant expressions of nestin were previously observed under malignant transformation [42].

In a recent study, nestin upregulation in infiltrating breast cancer cells with stem cell properties was found to be associated with the highly aggressive breast cancer subtypes [23, 24]. Moreover, in our study, high levels of nestin were present in tumor cells invading the stroma. Moreover, it has been shown that nestin-expressing progenitor cells in newly tumor-initiating populations might possess the capacity to differentiate not only into epithelial but also into endothelial cells which would be relevant for tumor growth and vascularization [25, 43]. Like in other tumors, a high rate of neovascularization is considered as poor prognostic factor in breast cancer [44].

Remarkably, higher numbers of MT1–nestin-coexpressing tumor cells invading the tissue stroma were related to a higher stage of disease, as (i) all six patients were at stages > I and as (ii) in this subgroup of six patients both stage III patients of the 17 quantified patients were included. Moreover, two patients had a high risk of relapse, if parameters like tumor size, presence of tumor cells in local lymph nodes, tumor differentiation grade, HER2 status and peritumoral vascular invasion (but not hormone receptors) were taken into consideration according to the St Gallen consensus 2005 [28]. Indeed, one patient died from distant metastases in the lung, brain and bone within 1 yr after primary diagnosis. By contrast, specimens without notable MT1–nestin coexpression in the invasive tumor derived from 3 patients being in a lower stage (< III) tumors, where two-thirds of the patients were still in stage I. However, these tumors were of the ER- and PR-negative and HER2-positive phenotype, which might explain that these patients had already an intermediate risk of relapse despite being in a low stage of disease. Despite these interesting findings, it should be mentioned that the sample size in our study was relatively low, so that further studies with a higher sample number are necessary to confirm our results.

In summary, this study revealed that MT1 is coexpressed with nestin in NCa and Ca in a considerable number of cells. High levels of MT1 in the Ca when compared with NCa specimens and the co-localization of MT1 and nestin in breast cancer cells invading the stroma were associated with a more advanced tumor stage and worse prognosis in a subset of patients. This warrants further studies on MT1-mediated signaling pathways in nestin-expressing breast cancer cells in order to elucidate the role and function of MT1 in the progression of breast cancer.

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Disclosure/conflict of interest

The authors have no conflict of interests.

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Expression of Melatonin – Metabolizing Enzymes and Cyclin D1 in Human Breast Cancer

Olga Rögelsperger, Katrin Wlcek, Cem Ekmekcioglu, Martin Svoboda, Robert Königsberg, Martin Klimpfinger, Walter Jäger, and Theresia Thalhammer

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EXPRESSION OF MELATONIN-METABOLIZING ENZYMES AND CYCLIN D1 IN HUMAN BREAST CANCER

OLGA RÖGELSPERGER[1], KATRIN WLCEK[2], CEM EKMEKCIOGLU[3*], MARTIN SVOBODA[1], ROBERT KÖNIGSBERG[4], MARTIN KLIMPFINGER[5], WALTER JÄGER[2] and THERESIA THALHAMMER[1]

Affiliation:

[1]Department of Pathophysiology, Center for Physiology and Pathophysiology, Medical University of Vienna, Vienna

[2] Department of Clinical Pharmacy and Diagnostics, University of Vienna, Vienna

[3] Department of Physiology, Center for Physiology and Pharmacology, Medical University of Vienna, Vienna,

[4] CEADDP Applied Cancer Research, Institution for Translational Research Vienna and Ludwig Boltzmann Institute for Applied Cancer Research, Vienna and

[5] Institute of Bacteriology and Pathology, Kaiser-Franz-Josef-Spital, Vienna, Austria

***Correspondence**

Cem Ekmekcioglu, MD

Department of Physiology

Center for Physiology and Pharmacology

Medical University of Vienna

Schwarzspanierstrasse 17

A-1090 Vienna, Austria

tel.: +43-1-4277-62114

fax.: +43-1-4277-62199

email: cem.ekmekcioglu@meduniwien.ac.at

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Abbreviations

Ca, cancer tissue; CYP, Cytochrome P450; ER, estrogen receptor; GPCR, G-protein coupled receptor; IHC, immunohistochemistry; IRS, immunoreactive score; NCa, non-carcinomatous tissue; RT-PCR, Reverse Transcription Polymerase Chain Reaction; SULT, Sulfotransferase.

Abstract

Background. Melatonin suppresses breast cancer cell proliferation by inhibiting the upregulation of estrogen-induced cyclin D1 via its G-protein coupled receptor MT1. Moreover, melatonin stimulates the expression of SULT1E1, an enzyme transforming estrogen into their inactive sulfates. However, metabolism of melatonin via 6-hydroxylation by CYP1A1/1A2 and subsequent sulfonation by SULT1A1/1A3 decreases its intracellular concentration, which would have a negative impact on its oncostatic action in breast cancer. *Patients and Methods.* We performed immunohistochemical (IHC) analysis of MT1 and cyclin D1 in breast cancer specimens from 33 patients. Also, we investigated the expression of CYP1A1/1A2, SULT1A1/1A3/1E1 and cyclin D1 in cancer (Ca) and adjacent non-cancer (NCa) specimens from 10 breast cancer patients using quantitative real-time RT-PCR. *Results.* CYP1A1-mRNA-expression was found only in 3 Ca and in one NCa. CYP1A2 mRNA was below the detection limit in all patients. SULT1A1 was observed only in 2/10 Ca and 1/10 NCa specimens. But all 10 Ca and NCa samples showed high SULT1A3 levels. Cyclin D1 mRNA- levels were found in all 10 Ca and NCa specimens. Furthermore, IHC-staining of cyclin D1 was observed in 27/33 Ca and correlated positively with estrogen receptor positivity (P=0.015). *Conclusion.* The low or even absent expression of CYP1A1 or CYP1A2 in breast cancer specimens, indicates that melatonin might be present in concentrations effective for cell cycle arrest, which warrants its use as a new target in breast cancer therapy.

Introduction

Breast carcinoma is worldwide the most common cancer form among women, accounting for around one third of all new cancer cases each year, and its incidence is still increasing [1]. Estrogens have been found to play an important role in the initiation and progression of this disease. They stimulate cell growth and inhibit apoptosis in the mammary gland via their binding to their nuclear estrogen receptor alpha (ER-α). Therefore, drugs which interfere with estrogen signaling, represent an effective adjuvant treatment of ER-α-positive breast carcinomas. Melatonin, an indoleamine produced by the pineal gland, was found to down-regulate ER-α and to block the binding of estradiol-ER-α-complexes to estrogen response elements (ERE) in DNA, resulting in suppression of estrogen-induced ER-α transcriptional activity on genes implicated in cell growth, division and survival [2]. One protein which is upregulated in response to estrogens and other mitogens and implicated in cell division is cyclin D1 [3]. Cyclin D1 forms complexes with cyclin-dependent kinases (CDK) 4 and 6, which are key regulators of the progression through the G1 phase of the cell cycle. Since aberrant activity of cyclin D1 results in functional inactivation of the retinoblastoma proteins which impede E2F transcription factors important for the initiation of DNA replication, cyclin D1 overexpression is associated with the development and progression of breast cancer, and occurs in up to 50 % of primary breast carcinomas [4].

In addition to its indirect effect on cell proliferation via interaction with estrogen signaling, melatonin directly induces cell cycle arrest in G0/G1-phase through blockade of the transcription factors ATF-2 and c-jun and their interaction with the cAMP-response element (CRE) of the cyclin D1 promoter [5]. The inhibition of cyclin D1

induction suggests melatonin as an effective agent also in the treatment of breast cancer being resistant to endocrine therapies.

In addition to its interference with estrogen signaling which is thought to be mainly mediated via activation of its G_i-protein coupled receptor MT1, melatonin acts as a potent antioxidant and free-radical scavenger protecting against DNA damage e.g. caused by oxidized estrogens [6, 7]

However, a limiting factor in the efficacy of melatonin action is its intracellular concentration which critically depends on inactivation by phase I cytochrome P450 isoenzymes (CYP) and phase II conjugation enzymes. Therefore, the local expression and activity of metabolizing enzymes is important (Figure 1).

As shown by studies on the hepatic metabolism of melatonin, its oxidation to 6-hydroxymelatonin, mainly catalyzed by CYP1A2 and CYP1A1, might play a role also in extrahepatic tissues [8]. The metabolite 6-hydroxymelatonin exhibits also antioxidant effects and has been demonstrated to bind to melatonin receptors MT1 and MT2, but to a lesser extent than its parent compound melatonin [7, 9]. However, conjugation to sulfate residues reduces binding of 6-hydroxymelatonin to its targets, enhances its hydrophilicity and therefore its excretion into urine. For the conjugation reaction, sulfotransferase SULT1A1 showing a high affinity particularly to 6-hydroxymelatonin, was found to be important in the sulfonation of 6–hydroxymelatonin in MCF-7 breast cancer cells [10]. Thus, the formation of 6-hydroxymelatonin sulfate represents a major pathway of melatonin metabolism, which is further supported by the fact that the measurement of urinary levels of 6-hydroxymelatonin sulfate accurately reflects plasma melatonin levels [11].

Previous studies demonstrated the expression of CYP1A1- mRNA and of CYP1A1- and CYP1A2- protein in tumor and adjacent histologically normal breast tissue by

quantitative RT-PCR and immunohistochemistry, respectively [12, 13]. Also, the expression of SULT1A1, SULT1A3 and SULT1E1 in malignant and surrounding non-malignant breast tissue was demonstrated [14]. SULT1A3 has been reported to be also involved in the biotransformation of 6-hydroxymelatonin, although to a lesser extent than SULT1A1 [15]. SULT1A3 further plays a role in conjugating the monoamines dopamine and serotonin, the precursor of melatonin in biosynthesis, to sulfate residues [16]. The enzyme SULT1E1 catalyzes the conjugation of estrogens to sulfate residues, and protects thereby breast cancer cells from excessive estrogenic actions [2]. Since melatonin was shown to stimulate the expression and activity of SULT1E1, SULT1E1 might be considered an important target in the antiproliferative action of melatonin [2]. In the present study, we assessed the expression of CYP1A1, CYP1A2, SULT1A1 and SULT1A3 considered to be relevant for the biotransformation of melatonin in breast tissue. In parallel, we also looked for the expression of MT1, cyclin D1 and the estrogen sulfotransferase SULT1E1, known targets for the oncostatic action of melatonin. For this purpose, we performed quantitative reverse transcription polymerase chain reaction on malignant and non-malignant tissue specimens obtained from 10 breast cancer patients. In addition, MT1 and cyclin D1 were analyzed immunohistochemically in breast cancer and the adjacent non-cancerous tissue specimens from 33 patients.

Patients and methods

Patients and samples

Frozen carcinomatous and adjacent histologically non-carcinomatous tissue and paraffin-embedded cancer tissue sections were used from 10 and 33 patients, respectively. All patients (mean age 59 ± 11 years; range: 40 – 94) were diagnosed with primary, invasive breast carcinoma at the Center for Oncology and Hematology of the Kaiser-Franz-Josef Spital, Vienna, Austria. The study protocol was approved by the ethical committee of the institution and written informed consent was obtained from all patients.

All samples were routinely subjected to pathological examination and only parts not required for further routine diagnosis were used for the study. In the routine diagnostic immunohistochemistry staining, 33 tumors were positive for ER-α, 16 for PR and 12 for HER2.

RNA Isolation, quantitative (TaqMan) reverse transcription-PCR

Frozen carcinomatous and adjacent non-malignant tissue from the same patient was used for the isolation of total RNA as described previously [17].

After reverse transcription of total RNA, real-time RT PCR was applied to evaluate cyclin D1, CYP1A1, CYP1A2, SULT1A1, SULT1A3 and SULT1E1 mRNA expression using the StepOnePlus system (Applied Biosystems) [17]. Quantitative real-time RT-PCR was performed in an amplification mixture with a volume of 20 µl. The target gene amplification mixture contained 10 µl 2X TaqMan® Universal PCR Master Mix, 1 µl of the appropriate Gene Expression Assay, 10 ng template cDNA dissolved in 5 µl nuclease free water, and 4 µl nuclease free water. β-actin was used as an endogenous control. All amplification experiments were done in triplicate. Sequence Detection

Software (SDS 1.9.1, Applied Biosystems) results were imported into Microsoft Excel, and comparable cDNA amounts in the specimens were calculated either according to the $2^{-\Delta\Delta Ct}$ method [18] or as described by Pfaffl [19].

Relative gene expression values are given as the n-fold change in transcription of target genes normalized to β-actin as endogenous control. The sample with the highest detectable target gene expression was arbitrarily used as calibrator, and the obtained data were calculated in relation to the calibrator's expression level.

Primers and probes

Quantitative Polymerase Chain Reaction (PCR) was performed with the following predesigned TaqMan® Gene Expression Assays containing intron-spanning primers (Applied Biosystems, Foster City, CA, USA): Cyclin D1: Hs99999004; CYP1A1: Hs01054797_g1; CYP1A2: Hs00167927_m1; SULT1A1: Hs00738644_m1; SULT1A3: Hs00413970_m1 and SULT1E1: Hs00193690_m1, β-actin: Part No 4326315E.

Antibodies

MT1: rabbit polyclonal antibody against human MT1 (Abcam, Cambridge, UK), 2.5 µg/ml; Cyclin D1: mouse monoclonal antibody against cyclin D1 (clone DCS-6, Dako, Glostrup, Denmark), 1:80 of cell culture supernatant. The peroxidase-conjugated Envision® kit (#K4065, Dako) was applied for the secondary antibody reaction.

Control experiments were done using rabbit anti-mouse IgG (H+L) (Pierce, Rockford, IL, USA) or mouse monoclonal IgG2a against Aspergillus niger glucose oxidase (Dako), instead of the primary antibody, respectively.

Immunohistochemical (IHC) detection

IHC staining of MT1 was performed as described [20]. Staining of cyclin D1 was done in an analogous manner, except for antigen retrieval, which was performed by boiling the sections for 10 min in 1 mM EDTA buffer (pH 8.0). The anti-cyclin D1 antibody was applied for 30 min (room temperature), whereas incubation with the anti-MT1 antibody was done overnight (5°C).

Light microscopy of the IHC-stained sections was performed with an Axioplan 2 microscope (Carl Zeiss, Jena, Germany), and images were recorded with an AxioCam HRc2 Color CCD digital camera (Zeiss) linked to the microscope.

Immunostaining of MT1 and cyclin D1 was assessed semi-quantitatively by two observers by scanning the whole section at 40x magnification to obtain immunoreactivity score values (IRS) between 0 (negative) and 12 (strong staining of >80% of cells) [21].

Statistical analysis

All statistical calculations and graphs were done with the SPSS 14.0 statistical software (Chicago, IL, USA). Data are expressed as mean $\pm$ standard deviation, and normally distributed data were compared by applying the two-sided unpaired t-test. Possible linear relationships between MT1- and cyclin D1- scores were assessed with Pearson correlation. For all tests, the level of significance was set to $p < 0.05$.

Results

Expression of CYP1A1-, CYP1A2-, SULT1A1- and SULT1A3 mRNA in malignant and non-malignant breast tissue

To get insight into the metabolism of melatonin in breast tissue, we analyzed the expression of the cytochromes CYP1A1 and CYP1A2, and also of the sulfotransferases SULT1A1 and 1A3 in malignant (Ca) and adjacent non-malignant (NCa) tissue specimens obtained from 10 patients with quantitative real-time RT-PCR.

CYP1A1 mRNA was found only in 3/10 patients (Figure 2b). CYP1A2 mRNA was below the detection limit in all 10 patients.

Expression of SULT1A1 was observed only in two Ca and one NCa specimens (Figure 2c). High mRNA levels of SULT1A3 were found in the Ca and NCa specimens from all 10 patients (Figure 2d). In 6 patients, SULT1A3 expression was 1.4 – 8.4 -fold higher in NCa than in the corresponding malignant specimen. By contrast, we found up to 4-fold higher SULT1A3 mRNA levels in the malignant than in the adjacent non-malignant specimens from four patients.

Expression of SULT1E1- mRNA in malignant and non-malignant breast tissue

SULT1E1, which transforms estrogens into their inactive sulfates, is an important target of melatonin action. We performed quantitative real-time RT-PCR analysis to investigate the expression of SULT1E1 in Ca and corresponding NCa specimens from 10 breast cancer patients. We found SULT1E1 expression only in 2 Ca and 2 NCa specimens from three patients (data not shown). High SULT1E1 mRNA levels in both, Ca and NCa, were seen in one patient.

Expression of cyclin D1- mRNA in malignant and non-malignant breast tissue

With respect to the role of melatonin in the inhibition of cyclin D1, we assessed the expression of cyclin D1 in malignant and non-malignant breast tissue.

Quantitative real-time RT-PCR-analysis showed high mRNA levels of cyclin D1 in all Ca and corresponding NCa specimens from 10 patients (Figure 2a). 4 patients showed up to 4.8-fold higher, and 4 patients up to 3.2-fold lower cyclin D1 expression levels in their Ca than the adjacent NCa. No difference in cyclin D1 mRNA levels between Ca and NCa was found for two patients. Since cyclin D1 is a downstream target of estrogen signaling, we assessed a possible relationship between cyclin D1 mRNA expression levels and ER-α status. We found significantly higher cyclin D1 levels in the five ER-α positive compared to the five ER-α negative Ca specimens (n-fold change: 0.65 $\pm$ 0.35 vs. 0.16 $\pm$ 0.10, P = 0.034).

Immunohistochemical analysis of cyclin D1 in breast cancer tissue sections

Due to the high expression of cyclin D1 mRNA in malignant breast tissue, we were also interested to take a look at the cyclin D1 protein level. As a consecutive next step, we conducted single IHC-staining of cyclin D1 on Ca tissue sections from 33 well-characterized breast cancer patients, and analyzed the percentage of stained cells and the staining intensity, from which we calculated the immunoreactive score (IRS) ranging from 0 to 12. Staining of cyclin D1 in cell nuclei was observed in 27 of 33 breast cancer sections, with a median IRS of 3. In concordance with PCR data, the IRS of cyclin D1 correlated positively with the ER-α-status (IRS: 4.6 $\pm$ 3.4 in ERα+ vs. 0.8 $\pm$ 1.6 in ERα-, P = 0.015) (Figure 3).

Immunohistochemical analysis of MT1 in breast cancer tissue sections

Since studies in breast cancer cell culture showed the involvement of MT1 in the antiproliferative effect of melatonin, we also investigated the expression of MT1 in breast cancer tissue sections obtained from 33 patients.

We observed cytoplasmic staining of MT1 in tumor cells in 31/33 Ca tissue sections subjected to single IHC-staining, with a median IRS of 8. With respect to melatonin's inhibitory effect in estrogen signaling, we also examined a possible relationship between MT1 IRS and ER-α status. However, MT1-scores were similar in ER-α-positive and –negative specimens. As shown in Figure 3, staining of both, MT1 and cyclin D1 was seen in 25/33 tumor specimens, but the immunoreactive scores of MT1 were not related to those of cyclin D1.

Discussion

In this study we examined the expression of enzymes of biotransformation relevant for the metabolism of melatonin in breast cancer and its adjacent non-malignant tissue. We found modest CYP1A1 mRNA expression in only three out of ten patients while, as expected, CYP1A2 mRNA was below the detection limit in all ten cases. Therefore, in our patient collective, melatonin might not be oxidized to 6-hydroxymelatonin, which is a prerequisite for conjugation to sulfate residues by SULT1A1 and 1A3. Moreover, SULT1A1 mRNA was expressed in three of ten patients being different to those with CYP1A1 expression. Since SULT1A1 is regarded the main responsible enzyme for the formation of 6-hydroxymelatonin sulfate, these data indicate that in our patient collective melatonin might be present in concentrations effective for its oncostatic action.

Recent studies showed two main pathways of melatonin metabolism in humans. In one case, melatonin's interaction with reactive oxygen species results in formation of N^1-acetyl-N^2-formyl-5-methoxykynuramine, while in the other case melatonin is oxidized to 6-hydroxymelatonin. The first pathway is found predominately in brain, while the second is important for circulating melatonin [7]. The latter reaction is mainly catalyzed by the phase-I-enzymes CYP1A1 and hepatic 1A2, as shown by Ma et al [8]. We found CYP1A1 mRNA only in three out of ten patients, were it was expressed in the Ca specimens and in one case additionally in the surrounding NCa tissue. Moreover, none of the 10 patients showed expression of CYP1A2 in their Ca or NCa specimens. This finding is in accordance with a previous study on human breast tissue showing no CYP1A2 expression as assessed by quantitative RT-PCR [12]. However, contrary to our results, Modugno et al found high levels of CYP1A1 mRNA in more than 90% of

both, Ca and adjacent NCa specimens, and an immunohistochemical study showed faint cytoplasmic staining of CYP1A2 in breast tumors [12, 13].

Due to our findings of modest CYP1A1 mRNA expression in only three and the lacking CYP1A2 expression in all examined patients, we hypothesize that melatonin might not be metabolized to 6-hydroxymelatonin in at least seven patients. A recent study from our lab clearly showed that formation of 6-hydroxymelatonin is a prerequisite for sulfonation by SULT1A1, since application of melatonin to SULT1A1-transfected MCF-7- and MDA-MB 231 cells resulted in only 1% of the amount of 6-hydroxymelatoninsulfate formed after incubation with 6-hydroxymelatonin [10]. Since the patients with SULT1A1 expression were not identical to those with CYP1A1 expression, melatonin might not be metabolized to 6-hydroxymelatoninsulfate in our examined patient collective. However, sulfonation of 6-hydroxymelatonin might be also partially catalyzed by SULT1A3, as shown by Honma et al [15]. Analysis of SULT1A3 expression in our patient collective showed considerable mRNA levels in Ca and NCa specimens from all ten patients. This implicates that a partial biotransformation of melatonin to 6-hydroxmelatonin sulfate might be possible in the three patients with CYP1A1 expression.

On the other hand we should also consider that polymorphisms in the genes encoding CYP1A1, CYP1A2 and SULT1A1 have been described in breast cancer, which might apply also for our patient collective [22]. Since the primers applied in our experiment specifically bind to one variant of the respective enzyme-encoding gene, a possible genetic polymorphism might be the reason for the low expression levels of CYP1A1, CYP1A2 and SULT1A1 in our patient collective. Remarkably, the different genotypes might show an altered enzymatic activity, resulting in either decreased inactivation or enhanced metabolic activation of drugs and mutagens. Besides possible

single nucleotide polymorphisms, hypermethylation in the SULT1A1 gene promoter has been described to occur in around 60% of breast cancer specimens, leading to transcriptional silencing of the SULT1A1 gene [23]. This finding might have also an impact on the sulfonation and hence deactivation of estrogens, because SULT1A1 was shown to be involved in the sulfonation of DNA-damaging catecholestrogens, but also of estradiol in high (micromolar) concentrations [15, 24].

Sulfonation of estrogens is an important mechanism for the regulation of estrogen activity, as estrogen sulfates do not bind to the estrogen receptor [24]. SULT1E1 shows a high affinity for estrogens at nanomolar concentrations and is therefore regarded the main estrogen metabolizing enzyme, thereby affecting the availability of the hormone in breast cells and consequently cell proliferation [24]. With respect to the role of SULT1E1 in controlling estrogen activity in breast tissue and due to the property of melatonin of stimulating the expression and activity of SULT1E1, we assessed mRNA levels of SULT1E1 in our patient collective [2]. However, SULT1E1 was below the detection limit in seven of ten patients, and high SULT1E1 mRNA levels in both, Ca and adjacent NCa tissue were observed only in one patient. Since our investigation showed only minute amounts of both, SULT1E1 and SULT1A1 mRNA levels in our patient collective, estrogens might not be sulfated and might therefore bind to their estrogen receptor, starting with the transcription of genes required for cell division, like cyclin D1.

Cyclin D1 was recently reported to be a target in the antiproliferative action of melatonin [5]. In MCF-7 cells, physiologic (nanomolar) concentrations of melatonin were shown to prevent the induction of cyclin D1 transcription via interaction with the cAMP response element binding site in the cyclin D1 promoter [5]. Because many

antiproliferative effects of melatonin at nanomolar concentrations are mediated via its G_i -protein coupled receptor MT1, we investigated whether the expression of cyclin D1 would correspond to that of MT1 [6]. Our PCR results show high cyclin D1 mRNA levels in Ca and NCa specimens from all patients. Four of ten patients had even three-fold higher cyclin D1 levels in their NCa as compared to their adjacent Ca. This finding seems at first glance contradictory, since cyclin D1 expression is associated with cell proliferation. However, we should consider that NCa parts were derived from breast cancer specimens, and although apparently healthy looking, changes in the expression pattern could already precede signs of macroscopic malignant transformation. In agreement with our PCR data, we found cyclin D1 immunostaining in 27 of 33 Ca specimens. As expected from the induction of cyclin D1 transcription by estrogen signaling, in both experiments, significantly higher cyclin D1 levels were found in ER-α positive than in ER-α negative tumors. Similar findings of cyclin D1-overexpression preferentially in tissues of ER-α-positive breast cancers, have been previously reported by using quantitative real-time PCR and immunohistochemistry, [4, 25].

In this study, 31 of 33 breast Ca specimens showed intense immunostaining of MT1 in cancer cells. Accordingly, previous immunohistochemical and immunofluorescence studies, including our own, reported high MT1 protein levels in breast cancer tissue specimens [26] [20]. In agreement with these studies, in our present investigation, we did not find any relationship between MT1 levels and ER-status.

In conclusion, the low or even absent expression of melatonin-metabolizing enzymes in breast cancer specimens, suggests that melatonin might be present in concentrations effective for its antiproliferative effect. This might be of relevance for the application of

melatonin as adjuvant drug with antioxidant, immunoenhancing and sleep-regulating properties in the management of breast cancer patients.

Disclosure/conflict of interest

The authors declare to have no conflict of interests.

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Figure legends

Figure 1. Biotranformation limits the antiproliferative effect of melatonin. Melatonin (Mel) induces cell cycle arrest of breast cancer cells through multiple mechanisms, resulting in downregulation of cyclin D1, which is responsible for G1-S-transition. These mechanisms include i) binding to the G_i-protein coupled melatonin receptor MT1, resulting in decreased levels of cyclic adenosine monophosphate (cAMP) required for interaction with the cyclin D1 promoter, ii) direct blockade of the transcription factor c-jun by melatonin, and iii) direct inhibition of binding of the estradiol (E2)-estrogen receptor-alpha (ER-α)-complex to estrogen-response elements in the cyclin D1 promoter. These effects depend on the intracellular concentration of melatonin. Therefore, enhanced biotransformation of melatonin due to expression of CYP1A1/1A2 and SULT1A1/1A3 in breast cancer cells decreases the intracellular melatonin levels due to formation of 6-hydroxymelatonin and its subsequent sulfonation and excretion.

Figure 2. Expression of (a) cyclin D1, (b) CYP1A1, (c) SULT1A1 and (D) SULT1A3 mRNA in cancer (grey bars) and adjacent non-cancer (shaded bars) tissue from 10 patients. Relative mRNA expression levels were determined by quantitative TaqMan RT-PCR, and the values were normalized to β-actin as endogenous control. mRNA values represent the average $\pm$ standard deviation of triplicates, and are given as the n-fold change to the sample with the highest detectable target gene expression (calibrator).

Figure 3. Immunohistochemical analysis of MT1- and cyclin D1- expression in tumor cells of invasive breast cancer sections obtained from 33 patients. The immunoreactivity score (IRS) of cyclin D1 (shaded bars) and MT1 (black bars) is depicted for each patient. Also, the estrogen receptor (ER)- α status is given for each patient. The IRS was calculated by multiplicating the percentage of stained tumor cells with the staining intensity, as described in the methods part. IRS=0, no staining; IRS=12, intense staining of > 80% of tumor cells.

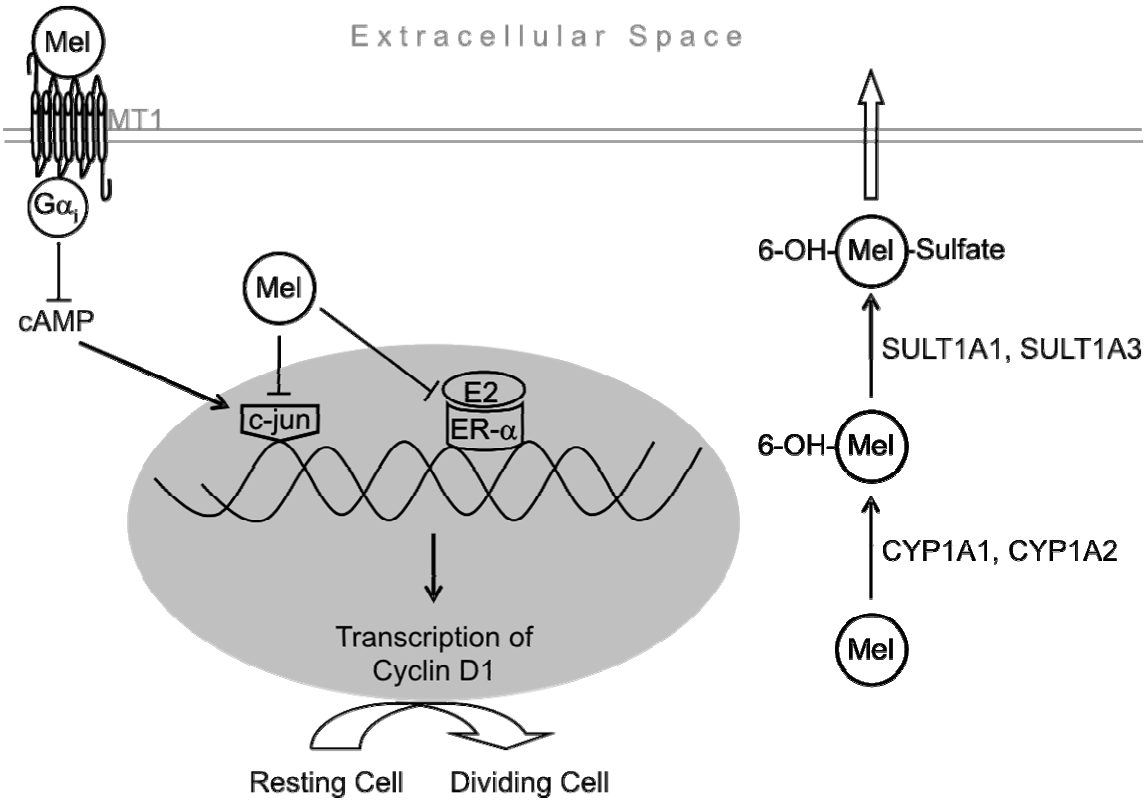
Extracellular Space
Mel
MT1
Gαi
cAMP
Mel
c-jun
E2
ER-α
Transcription of
Cyclin D1
Resting Cell
Dividing Cell
6-OH-Mel-Sulfate
SULT1A1, SULT1A3
6-OH-Mel
CYP1A1, CYP1A2
Mel

Figure 1.

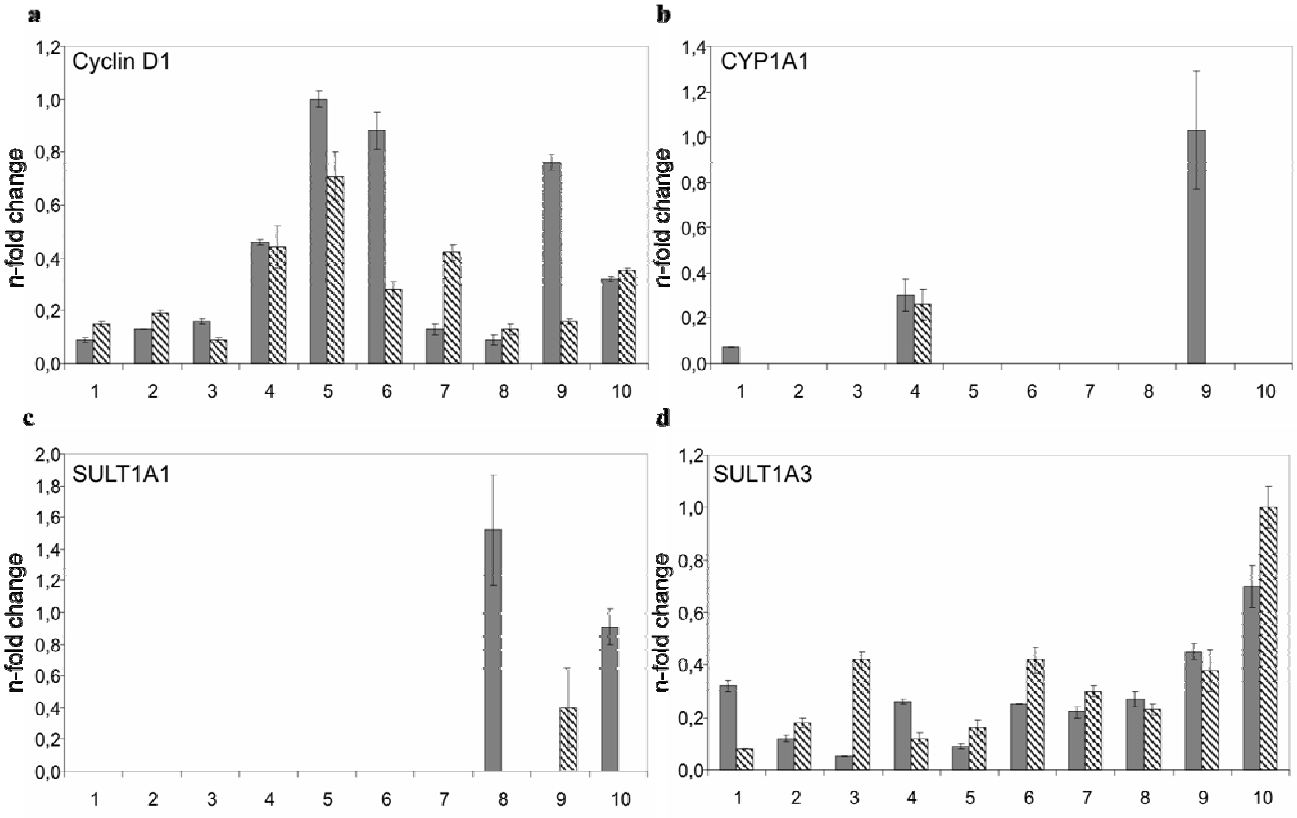
a
Cyclin D1
n-fold change
b
CYP1A1
n-fold change
c
SULT1A1
n-fold change
d
SULT1A3
n-fold change

Figure 2.

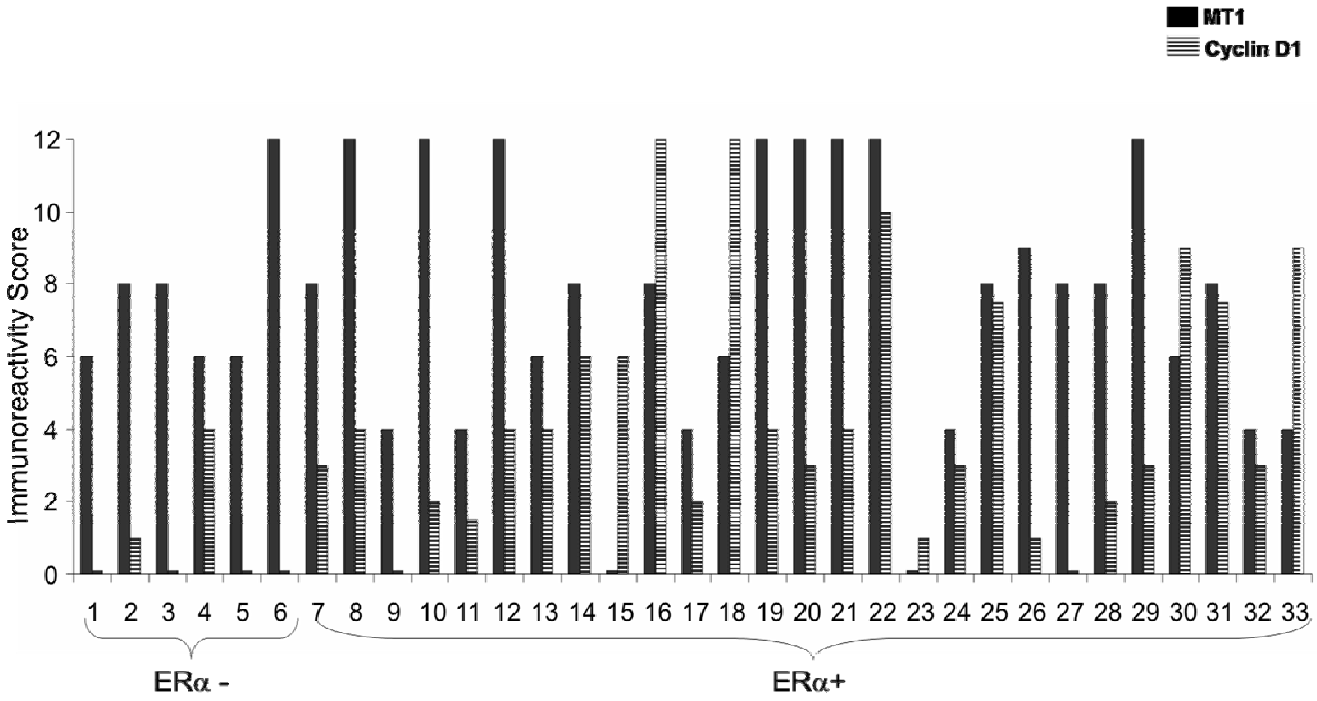
MT1
Cyclin D1
Immunoreactivity Score
0
2
4
6
8
10
12
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
ERα -
ERα+

Figure 3.

6 CURRICULUM VITAE

➢ Personal Data

Date of Birth: 1978-08-11

Place of Birth: Vienna, Austria

Parents: Rögelsperger Peter, horologist,
Rögelsperger Ingrid, clerk (retired)

Nationality: Austrian

➢ Education

1988 – 1996: Private grammar school with emphasis on economics, Kenyongasse 4-12, 1070 Vienna

1996, May: Leaving Exam (Matura)

1996, Oct – 2003, Mar:

Pharmacy - Studies at the Faculty of Natural Sciences, University of Vienna, Austria; academic degree: “Magistra Pharmaciae” (Mag.pharm.)

2000, Feb – Jul:

Diploma Thesis at the Department of Medicinal Chemistry, University of Perugia, Italy (ERASMUS exchange program), Title: “Evaluation of the Hydrophilic/Hydrophobic Balance of Bile Acids by Different Reversed Chromatographic Stationary Phases”

2003, May – 2004, May:

Aspirant Year (Aspirantenjahr) at the chemist’s shop “Elisabeth-Apotheke”, Favoritenstr. 76b, 1100 Vienna

2004, May: State exam to become a Licensed Pharmacist

2004, Jun – 2009, Jun:

PhD in Pharmacy at the Department of Pathophysiology, Medical University of Vienna, in collaboration with the Department of Clinical Pharmacy and Diagnostics, University of Vienna, Title: “Expression of Cell Cycle Regulators and Melatonin Receptor 1 in Human Breast Cancer Tissue: Relevance for Therapy”

➢ Employment History

Chemist's Shop:

1999, Feb and Jul:

Undergraduate Practician at the Germania-Apotheke (Mag.pharm. Fischill), Hütteldorferstr. 76, 1150 Vienna

2001, Feb – May:

Undergraduate Practician at the Apotheke "Zum Heiligen Florian" (Mag.pharm. Maruna), Wiedner Hauptstr. 60a, 1040 Vienna

2003, May – 2004, May:

Graduate Practician (40h / week) at the Elisabeth-Apotheke (Mag.pharm. Grassberger), Favoritenstr. 76b, 1100 Vienna

2004, Jun – 2005, Aug:

Part-time employment (12h / week) as registered Pharmacist at the Elisabeth-Apotheke, Favoritenstr. 76b, 1100 Vienna

Since 2005, Sep:

Part-time employment (8h / week + night service) as registered Pharmacist at the Marien-Apotheke (Mag.pharm. Weeber), Hartlgasse 25, 1200 Vienna

Industry and Wholesalers:

1997, Aug:

Practician at the pharmaceutical wholesaler "Herba Apotheker AG", Michelbeuerng. 9a, 1090 Vienna

2000, Aug and 2001, Aug:

Practician in laboratory of chemical synthesis at the research institute of NOVARTIS (Univ.Prof. Dr. Berner), Brunnerstr. 59, 1235 Vienna

University/Teaching Experience:

Since 2004, Oct:

Twice a year, tutor at the laboratory course in qualitative analysis of inorganic chemistry for undergraduate students of pharmacy (Univ.Prof. Mag. Dr. Jäger), Department of Clinical Pharmacy and Diagnostics

Since 2005, Jan:

Once a year, tutor at the laboratory course in qualitative analysis of inorganic chemistry for undergraduate students of Fachhochschule für Biotechnologie (Univ.Prof. Mag. Dr. Jäger), Department of Clinical Pharmacy and Diagnostics

➢ Awards

2008, Apr: Trainee Travel Award from the Awards Committee of the 10th Gordon Research Conference on Pineal Cell Biology – Mechanisms of Circadian Rhythmicity and Melatonin Action

➢ Skills

Languages: German (Mother tongue), English, Italian (both linguistic levels C1)

Statistics Two courses at the Medical University of Vienna with emphasis on Biostatistics and Survival Analysis, SPSS

Certificate for the TissueFaxs Cell Analysis Software, Tissue Gnostics GmbH, 1020 Vienna

Communication Skills Training at the Institute of Pathophysiology, Medical University of Vienna

Presentation Technique Course at Wirtschaftsförderungsinstitut (WIFI), Währinger Gürtel 97, 1180 Vienna

7 LIST OF SCIENTIFIC PUBLICATIONS

➢ Original Manuscripts

Königsberg R, **Rögelsperger O**, Jäger W, Thalhammer T, Klimpfinger M, DeSantis M, Hudec M and Dittrich C. *Cell Cycle Dysregulation influences Survival in High Risk Breast Cancer Patients.* Cancer Invest. 2008 Aug; 26(7): 734-40.

Rögelsperger O, Ekmekcioglu C, Jäger W, Klimpfinger M, Königsberg R, Krenbek D, Sellner F and Thalhammer T. *Co-Expression of the Melatonin Receptor MT1 and Nestin in Human Breast Cancer Specimens*. J Pineal Res. 2009 May; 46: 422-32.

Rögelsperger O, Wlcek K, Ekmekcioglu C, Svoboda M, Königsberg R, Klimpfinger M, Jäger W and Thalhammer T. *Expression of Melatonin-Metabolizing Enzymes and Cyclin D1 in Human Breast Cancer*. Submitted for publication to Acta Oncol.

➢ Poster-Presentations

Svoboda M, **Rögelsperger O**, Wlcek K, Liedauer R, Jäger W and Thalhammer T. *Organic Anion Transporters (SLCO/OATP) in paclitaxel sensitive and resistant ovarian cancer cell line A2780 and A2780-ADR*, 3rd PhD Symposium, Medical University of Vienna, June 2007.

Svoboda M, **Rögelsperger O**, Wlcek K, Liedauer R, Jäger W and Thalhammer T. *Paclitaxel resistance in the adriamycin-resistant ovarian cancer cell line A2780: Role of SLCO uptake transporters*, 5th International Symposium on Targeted Anticancer Therapies, Amsterdam, March 2007.

Svoboda M, Liedauer R, **Rögelsperger O**, Wlcek K, Jäger W and Thalhammer T. *Multidrug Resistance Transporters in Ovarian Cancer Cells*, 6th International Symposium on Minimal Residual Cancer, University of Hamburg, September 2007.

Rögelsperger O, Svoboda M, Huber S, Krenbek D, Königsberg R, Klimpfinger M, Jäger W and Thalhammer T. *Characterization of Melatonin Receptor (MT1) Expression in Human Primary Breast Cancer Specimens*, Joint Meeting of the Slovak Physiological Society and the Physiological Society and the Federation of European Physiological Societies, Bratislava, September 2007.

Rögelsperger O, Ekmekcioglu C, Svoboda M, Königsberg R, Klimpfinger M, Jäger W and Thalhammer T. *The Impact of the Melatonin Receptor (MT) 1 on Breast Cancer Progression*, The Gordon Research Conference on Pineal Gland Biology, Barga (Lucca), April 2008.

Rögelsperger O, Ekmekcioglu C, Svoboda M, Königsberg R, Klimpfinger M, Jäger W and Thalhammer T. *Melatonin Receptor (MT)1 and Nestin Coexpression: an Indicator of More Advanced Breast Cancer?*, EACR20 – 20th Meeting of the European Association for Cancer Research, Lyon, July 2008, and Symposium of the Center for Physiology, Pathophysiology and Immunology of the Medical University of Vienna, September 2008.

Krenbek D**, Rögelsperger O**, Ellinger I and Thalhammer T. *Expression of the Melatonin Receptors MT1 and MT2 in Human Hepatocellular Carcinoma Cells*, Symposium of the Center for Physiology, Pathophysiology and Immunology of the Medical University of Vienna, September 2008.

- Oral Presentation

The Melatonin Receptor MT1 and the Intermediate Filament Protein Nestin are Co-Expressed in a Subgroup of Human Primary Breast Cancer, 4th PhD Symposium, Medical University of Vienna, 28. – 29. May 2008.