Oncogenic pathways in lobular breast cancer

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Oncogene signaleringsroutes in lobulaire borstkanker

(met een samenvatting in het Nederlands)

Lobüler meme kanserinde onkojenik sinyal yolları

(Türkçe Özet ile)

Proefschrift

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Promotoren; Prof. dr. P.J. van Diest
Prof. dr. E. van der Wall

Co-promoter; Dr. P.W.B. Derksen

Ankara'daki Ailem'e...
Bana verdikleri sonsuz sevgi ve koşulsuz destek için.

To my family in Ankara...
For their loving and unconditional support.

Contents

Chapter 1

General Introduction

Breast cancer is the most frequent cause of cancer related death occurring among women (World Cancer Report, 2008) in both developed and developing countries. It affects approximately 1 in 8 women in the Western world with a total of more than one million new cases worldwide per year, of which 35% will eventually die. Breast cancer is a heterogeneous and complex disease with several histological and molecular characteristics within tumors and between patients [1]. Invasive ductal carcinoma (IDC) is the most common form of breast cancer (~80%), while 10%–15% of patients present with invasive lobular carcinoma (ILC), and the rest comprises rare subtypes [2, 3]. Lobular and ductal carcinomas differ from each other in terms of molecular features, histology and response to therapy (detailed information in Chapter 2). Histologically, classic ILCs are characterized by small and round shaped cells that infiltrate individually or in strands, with frequently intracytoplasmic vacuoles and small to intermediate size nuclei. The discohesive tumor cells are highly dispersed and locally very invasive, making diagnosis with physical examination and mammography difficult [4]. Therefore, ILC is relatively often an incidental finding on a breast biopsy taken for other clinical or radiological mild abnormalities.

ILCs do more frequently express hormone receptors than IDCs do [5] and generally respond to endocrine therapy. However, ILCs with high histological grade usually loose the hormone receptor expression and therefore become resistant to endocrine therapy [6]. Compared to IDC, ILC more frequently shows low/absent EGFR and HER-2 expression [2] resulting in a lower eligibility for anti-EGFR family monoclonal antibody or small molecule based therapies (e.g.CetuxiMab, Lapatinib, Herceptin) [7, 8]. It has been reported several times that ILC has a worse prognosis than IDC and this is a clear sign of inadequate success rate of current treatment strategies against this malignancy [5, 9, 10]. ILCs exhibit certain molecular aberrations such as loss of chromosome 16q and lack of E-cadherin expression which are characteristic to this subtype [11]. Nevertheless, there is a lack of further understanding in the molecular pathways and abnormalities associated with lobular cancer etiology.

Aim of the thesis

A comprehensive understanding of the molecular pathways and aberrations leading to ILC development and progression is required to improve the existing strategies and to develop novel therapies. Several signaling pathways which play a key role in normal mammary gland development and homeostasis have been identified, and increasing evidence indicates that the same pathways are often deregulated in breast cancer. The aim of this thesis was to study the involvement of some of these key pathways in lobular breast cancer growth and maintenance to gain a better insight in this breast cancer subtype. Eventually, this investigation could reveal attractive candidates for additional treatment options for individualized treatment regimens of patients with ILC.

Scope of the thesis

In **Chapter 2,** molecular, pathological and clinical characteristics of lobular breast cancer are extensively reviewed. **In Chapter 3**, an overview of the current literature

on the role of mammary stem cells is given in the context of normal breast and breast cancer development. This review offers broad information on the key molecular pathways controlling normal mammary homeostasis and playing a role in breast cancer progression, with emphasis on the NOTCH pathway which is frequently altered in human breast cancer. **Chapter 4** investigates the role of NOTCH signaling as a possible therapeutic target in the growth and maintenance of lobular breast cancer. In this chapter, frequent NOTCH activation in lobular breast carcinomas and response to NOTCH inhibitors is described. In addition, we investigated associations between NOTCH and HIF-1α signaling in human breast cancer specimens and metastases in **Chapter 5.** These two highly conserved signaling pathways are known to play a role in normal development and breast cancer and show interplay *in vitro* where hypoxia induced NOTCH activation through HIF transcription factors.

Chapter 6 is devoted to investigating mutations in the tumor suppressor p53 in two different subtypes of lobular breast cancer; classic and pleomorphic. In **Chapter 7**, we investigated copy number changes of tumor suppressor and oncogenes in classic and lobular breast cancer cases. Finally, **Chapter 8** summarizes and discusses all the findings reported in this thesis.

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Chapter 2

Lobular Cancer of the Breast; A Review

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Manuscript

Abstract

Lobular breast cancer is a subtype of breast cancer comprising 10-15 % of all breast cancer cases. The clinical diagnosis of this subtype remains to be difficult whereas its incidence has been increasing. The histopathological characteristics of this disease have probably been better defined than its biological features. Here, we review the pathological and molecular features of lobular cancer.

1. Introduction

1.1 Terminology & History

Lobular carcinoma accounts for 10-15% of all breast cancer cases [1] and is the second most common histological type of breast cancer. Two microphotographs documented by Ewing in 1919 were the first documentation of this subtype (Ewing J. Neoplastic diseases: a textbook on tumors). Two decades later, in 1932, it was Broders who defined lobular "carcinoma in situ" (LCIS) for the first time (Broders, A. C. Carcinoma in Situ Contrasted with Benign Penetrating Epithelium. J. Am, Med. Assoc, 99: 1670-1674, 1932.). Later, in 1942, Foote and Stewart described this form of breast cancer as a population of small monomorphic cells filling and distending from the terminal ducto-lobular unit (where early progenitor cells determine the histological fate) [2] spreading in a pagetoid manner through the ductal system. In time, less extensive lesions within LCIS were noticed and called as atypical lobular hyperplasia (ALH) (Muir R. The evolution of carcinoma of the mamma. J. Pathol. Bacteriol. 1941). The term lobular neoplasia (LN) was introduced only later by Haagensen [3] who used this word to cover the spectrum of disease; from ALH to LCIS. So far, LN has been described not only as often associated with invasive lobular breast cancer but also as a risk factor for subsequent development of invasive cancer [4-6]. This hypothesis is supported by increasing molecular evidence which show that LCIS is a likely precursor of invasive lobular cancer (ILC), which will be discussed later in this review [7].

1.2 Subtypes & Morphology

1.2.1 Lobular neoplasia

As it has been described by Rosen et al. (Rosen PP, Oberman H. Tumors of the Mammary gland. Washington, DC, Armed Forces Institute of Pathology, 1993), ALH is as an intralobular proliferation of discohesive fairly monotonous small cells with scant cytoplasm and often clear cellular borders, with often vacuoles in the cytoplasm (to form "signet (ring) cells"), mildly distending the individual acinar structures but not completely solidifying them (often less than half of the acinar structures of the lobule). The nuclei are fairly monotonous, small, rounded, have bland chromatin and no or small nucleoli. Necrosis is absent and mitoses and apoptosis are rare. LCIS is more outspoken with regard to these criteria: more monotonous and completely solidifying more than half of the acinar structures.

In view of the problems with differentiating between ALH and LCIS and the wide overlap in molecular features, ALH and LCIS are nowadays grouped as LN (Figure 1A & B). Although usually located in the lobuli, LN cells may spread to the ductal system, denoted "Pagetoid spread" [4, 8, 9]. Very often, LN is diffusely found in the affected breast. High grade variants of LN are pleomorphic LCIS with very atypical nuclei and sometimes apocrine differentiation, and the macroacinar type with hugely distended acini that tend to become necrotic in the center because of severe hypoxia, and may contain microcalcifications [11] (Figure 2). These high grade forms of LCIS are more difficult to discern from ductal carcinoma *in situ* (DCIS) and tend to behave more as

DCIS in the sense that they usually form more localized disease that is easier treatable with surgery.

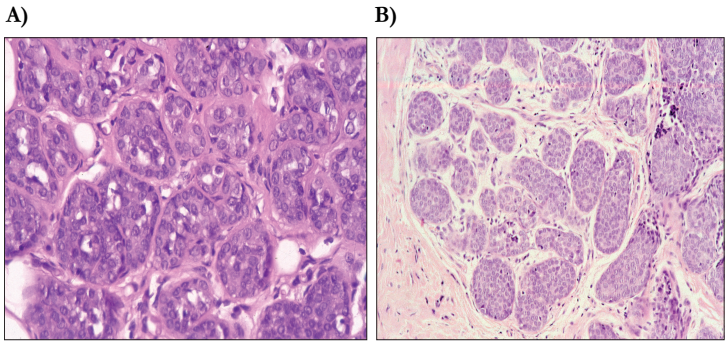

Figure 1. *Representative images of atypical lobular hyperplasia (A) and lobular carcinoma in situ (B).*

Alternatively, LN (here: lobular intraepithelial neoplasia (LIN)) has been divided into three groups based on different degrees of atypia; LIN1, LIN2 and LIN3 [10]. LIN1 is comparable to ALH, LIN2 to classic (low grade) LCIS, and LIN3 to pleomorphic or macroacinar LCIS.

16 There is increasing molecular evidence showing that LCIS is a likely precursor of ILC. For example, conventional cytogenetic and comparative genomic hybridization (CGH) analysis revealed recurrent genomic gains on chromosome 1q and loss of chromosome 16q in ALH, LCIS and ILC highlighting their common origin (see further below) [11-15].

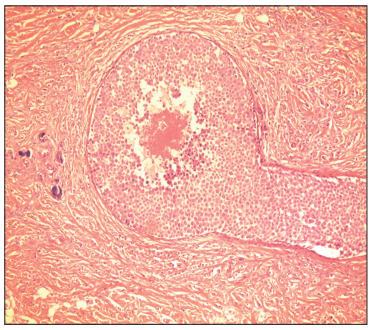

Figure 2. *Macroacinar LCIS with central necrosis, surrounded by ILC*

1.2.2 Invasive lobular cancer

The classic type of ILC is characterized by small regular cells with frequently intracytoplasmic vacuoles, small rounded nuclei with no or small nucleoli, discohesively infiltrating as single cells or one layer thick ("indian") files in a targetoid pattern around uninvolved ducts (Figure 3 A & B). The mitotic rate is low and there are few apoptotic cells.

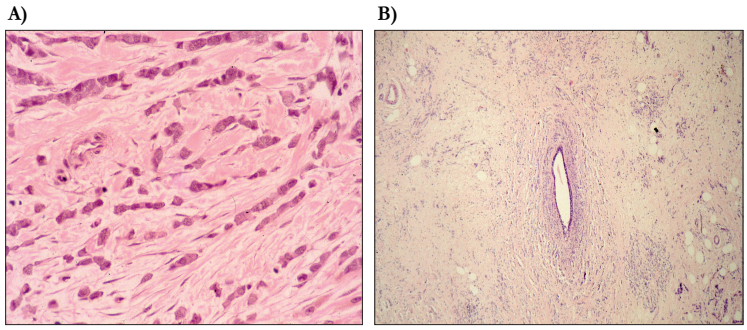

Figure 3. *Representative images of classic type invasive lobular cancer (A) and invasive lobular cancer with targetoid growth (B).*

Some ILC variants have been described which have a different architecture but classic lobular cytonuclear appearance (the alveolar and solid subtypes), or which have a classic architecture but different cytonuclear appearance (the pleomorphic, myoid and histiocytic subtypes). The alveolar type is defined by its typical lobular cytology but architectural pattern of round aggregates of 20 or more cells (Figure 4A).

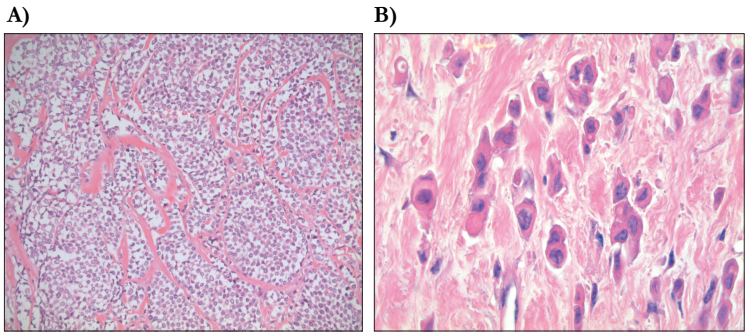

Figure 4. *Representative images of ILC with alveolar architecture (A) and ILC with apocrine cellular differentiation (B).*

Solid ILC shows solid sheets of lobular type cells with little intervening stroma. This variant often exhibits more mitoses and more nuclear atypia. The pleomorphic variant of ILC displays cells with polygonal, highly atypical nuclei and also more frequent mitoses, infiltrating in a classic lobular pattern [16].
The myoid, histiocytic and apocrine subtypes (Figure 4B) are composed of polygonal larger cells with distinct eosinophylic cytoplasm resembling striated muscle cells, histiocyt-like cells and apocrine cells, respectively.

1.3 Incidence

LCIS is found in about 5% of all cancer excision specimens [17, 18]. The incidence of LCIS appears to have increased in the last two decades [19] as stated by a study based

on Surveillance, Epidemiology, and End Results (SEER) data. This increase is likely to be due to the improvement of screening methods and its widespread use together with the increased consciousness of the pathologists about this lesion [20, 21].
ILC is known to comprise approximately 10-15% of all breast cancers; the study of Orvieto et al. gives an idea about the frequency of ILC subtypes [22]. In their report, the investigated 530 patients and observed that 57% classic, 19% alveolar, 11% solid, and 13% pleomorphic, signet ring cell, histiocytoid, or apocrine features.

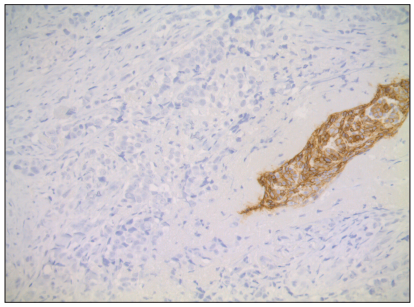

Figure 5. *ILC with complete loss of E-cadherin expression surrounding a pre-existent duct with normal expression*

2 Molecular Features

2.1 Immunophenotype

LN and most of its variants have a similar immunophenotype. They usually express the estrogen (ER) and progesterone receptors (PR), and only rarely overexpress HER2. They lack cytokeratin (CK)5, CK14 and EGFR expression, usually negative for p53, but express CK18 [1, 34, 35]. Exceptions are pleomorphic LN and ILC that are more often ER/PR negative but HER2 and p53 positive [23], and apocrine LN and ILC that are more often ER/PR negative but HER2 positive, and express GCDFP15 and the androgen receptor (AR). E-cadherin expression is very often lost or at least diminished (see further below) (Figure 5). Thereby, most LN and ILCs are considered to be "luminal A" lesions, pleomorphic and apocrine LN and ILC being exceptions belonging rather to the HER2 driven or Luminal B type spectrum.

2.2 Genetic changes

Over the last decades, investigations on molecular features that are characteristic to certain subtypes of breast cancer have led to increased understanding on molecular evolution and progression of breast cancer. Genetic data based on conventional cytogenetic and comparative genomic hybridization (CGH) analysis showed no significant difference between ALH and LCIS. Furthermore, these data demonstrated that LCIS/ILC harbor recurrent genomic gains on chromosome 1q and loss of 16q which are also features of low grade DCIS/infiltrating ductal cancer (IDC) [11-14, 24]. These reports revealed LCIS as a direct precursor to ILC. Moreover, the results also supported a common evolutionary link between LCIS and low grade DCIS/IDC [25]. The recurrent loss of chromosome 16q in ALH, LCIS and ILC supports the common evolutionary origin of

these three conditions [15, 26].
The E-cadherin gene (*CDH1*) is located on chromosome 16q and mainly known by its role in cell adhesion. Human *CDH1* was cloned and characterized by Bussemakers et al. [27] who later identified inactivating somatic mutations in *CDH1* in lobular breast carcinomas [28, 29]. Interestingly, Guilford et al. identified a germline mutation in *CHD1,* the locus for E-cadherin, as a hereditary factor in families with both gastric and breast cancer [30]. In cases where LCIS and ILC co-existed, similar truncating mutation have been observed in both of them, which is more evidence supporting their common origin [31, 32].
DCIS (especially high grade) seems to develop through divergent molecular pathways than LN/ILC since it harbors very distinct genetic changes compared to LN/ILC such as chromosomal alterations in 6q, 8q, 11q and 17q [33, 34]. Low grade DCIS and LN appear to have a stronger evolutionary association and high grade DCIS seem to be the most distinct one. Interestingly, several reports indicated that pleomorphic variant of LN/ILC show similar characteristics to high grade DCIS which certainly added to the complex nature of evolutionary associations of breast cancer subtypes [35-37]. Eusebi et al. reported the presence of apocrine differentiation in a group of pleomorphic ILC cases based on the positive expression of gross cystic disease fluid protein 15 (GCDFP-15) in these cases. GCDFP-15 is encoded by the *PIP* gene. Moreover, amplifications/overexpression of HER2 and accumulation/mutations of p53 in pleomorphic ILC were reported [35]. Partial gains and deletions of chromosomes (1p, 8p, 12p, 14q, 18q, 19p+q) and gain of oncogenes (e.g. c-myc, HER2) are among the similarities of pleomorphic LN/ILC and high grade DCIS [38]. However, recent reports demonstrated that only a very small percentage of genes were differentially expressed between classic and pleomorphic ILC, indicating that pleomorphic LN/ILC are different from DCIS/IDC [39, 40]. Pleomorphic ILC cases showed a lack of E-cadherin and β-catenin expression and gain of 1q and loss of 16q, which are characteristics of ILC but not IDC.
In conclusion, these results point to the concept of pleomorphic variant emerging via a common genetic pathway shared by DCIS/IDC and LN/ILC [41]. Allred et al. also reveal that ILC has an exclusive genetic profile compared to their grade and molecular subtype matched IDC, mostly on genes involved in processes like adhesion and cytoskeletal signaling. Considering the characteristic morphology of LN/ILC, this was not an unexpected observation. Today, the most characteristic molecular feature of LN and ILC remains the frequent loss of E-cadherin [32, 42] (see below).
Together, these data support the progression routes described in Figure 1. ILC is mainly thought to derive from normal breast through stages of ALH and LICS. Columnar cell lesions are also thought to progress to ILC through stages of CCL with hyperplasia and atypia. Thirdly, also some forms of low grade DCIS may be precursors of ILC (Figure 6).

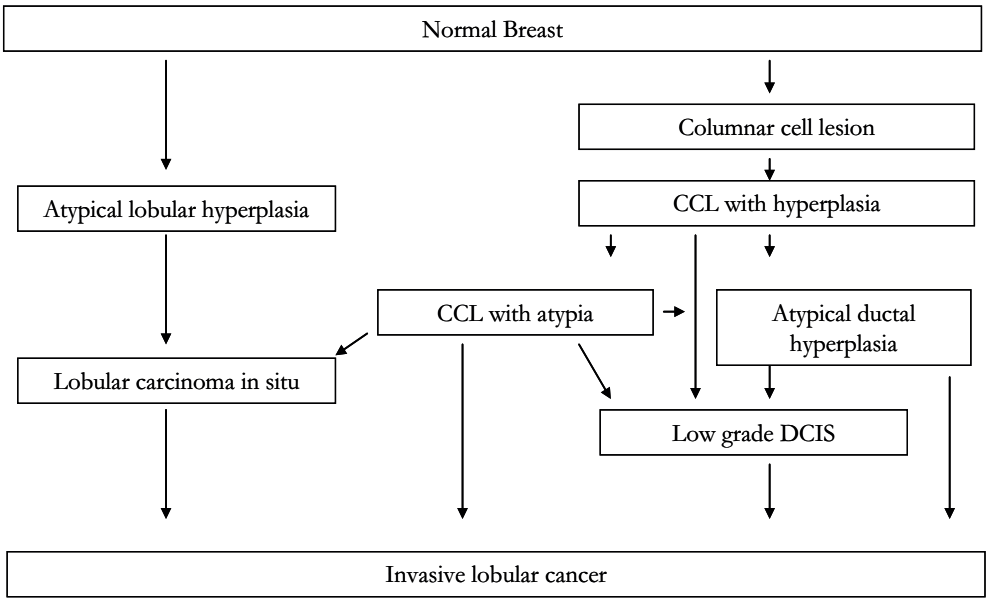

Figure 6. *Schematic representation of progression routes from normal breast to ILC*

3 Clinical Diagnosis

LN is usually too small and too scattered to present as a palpable lesion, and similarly does not show up on imaging (except for calcified macroacinar LN). Pure LN is thereby almost always an incidental finding, made after pathological examination of a breast biopsy taken for other clinical or radiological abnormalities. This makes it difficult to estimate the exact incidence of the disease since there are certainly women who do not show any symptoms. According to studies reporting the clinical, pathological and mammographic features of LN, various types of microcalcifications with additional opacities suggestive of malignancy are associated with LCIS [43-46]. Microcalcifications were observed usually around the areas of adenosis and apocrine metaplasia near the tumor area but not inside it (except for the macroacinar type). In addition, frequent presence of sclerosing adenosis of mastopathy, and lobular hyperplasia was observed. Fibroglandular type density was detected in the breasts of patients with LN. Multifocality in the ipsilateral breast is observed in more than 50% of the patients diagnosed with LCIS and 30% of these cases develop LCIS in the contralateral breast as well [47]. The diagnosis of ILC with physical examination, mammography, sonography, MRI and PET scanning is also often difficult due to the lack of characteristic radiological abnormalities and the fact that also ILC often does not present as a palpable lump [48-52]. MRI has been shown to have the highest sensitivity of current imaging techniques for ILC. In a more recent study, Brem et al (2009) investigated and compared the sensitivity of mammography, sonography, MRI, and breast-specific gamma imaging (BSGI) in the

detection of ILC [53] and concluded that there was no statistically significant difference among these techniques.
In comparison to IDC patients, ILC patients are more likely to be older, and the ILC tumors tend to be larger in size [1]. More often than in IDC, there is diffuse or multifocal infiltration, therefore breast conserving surgery and heat ablation strategies [54] are more likely to be irradical.

4 Prognosis

4.1. Lobular neoplasia

The relative risk to develop subsequent invasive cancer was originally estimated to be 4-5 fold for of ALH and 8-10 fold for LCIS, which is in the same order as atypical ductal hyperplasia (ADH) and DCIS, respectively [55].
Multifocality in the ipsilateral breast is observed in more than 50% of the patients diagnosed by LCIS and 30% of these cases develop LCIS in the contralateral breast as well [47]. The latter case is still a point of discussion since there are conflicting reports on the risk of contralateral breast cancer associated with LCIS [56, 57]. In these two studies based on a SEER data; Chuba et al. reported an equal number of contralateral and ipsilateral invasive cancers occurring in women with LCIS; however, Li et al. reported that ipsilateral cancers were the most frequently observed. Irrespective of their conflicting findings, both studies showed that a large proportion of women with a history of LCIS developed ILC. A third study observed that the incidence of contralateral breast cancer in women with ILC was higher than in women with IDC [1]. Arpino et al. [58] did a survey on core biopsies which were diagnosed with LCIS or ALH to determine the incidence of breast carcinoma in these patients. The follow-up excisional biopsies of these women were investigated and invasive disease was found in 14% of those that were initially diagnosed with LN only. Therefore the investigators concluded that surgical biopsy is needed for establishing the presence of LCIS. In view of the above described multifocality and high rate of ipsilateral invasive recurrences, the proper surgical treatment for LN would be bilateral mastectomy, but this is generally considered massive overtreatment.
There are relatively few validated prognostic factors for LN. Bodian at el. stressed the importance of age at diagnosis of a benign lesion or LCIS and family history as reliable prognostic factors. These factors appeared to reflect poor prognosis for this malignancy [59]. Both of these factors were previously reported by London and Haagensen et al. as well [3, 60]. Furthermore, the number of lobules with LCIS on the specimen and nuclear size were shown to be significantly related with recurrence of the disease [61].

4.2 Invasive lobular cancer

Reported comparisons between the prognosis of ILC vs. IDC are inconsistent; some studies report that ILC has a better prognosis [62], some conclude that there is no difference [63] and some states it has worse prognosis [64, 65]. However in all these studies, only very small groups of patients were included. Recently, Arpino et al. [1]

confirmed the lack of prognostic difference between these two biologically different entities in a very large group of 4140 cases with ILC and 45169 cases with IDC.
Hormone receptor positivity is a prognostic factor in ILC [66]. Orvieto et al. [23] pointed out the prognostic role of histopathologic subtyping of ILC since non-classic subtypes of ILC had poorer prognosis.

5 Treatment

5.1. Lobular neoplasia

In the past, mastectomy was a frequently applied management strategy for the case of LCIS (Ewing J. Neoplastic diseases: a textbook on tumors. Philadelphia, PA: WB Saunders; 1919) [67]. Bilateral mastectomy was also suggested as a possible management strategy with the claim that LCIS gives rise to invasive cancer in both breast with an equal risk [68, 69]. However, in the light of recent investigations showing only a small percentage of women being diagnosed with invasive carcinoma in the contralateral breast, the management of this malignancy has shifted towards a more conservative approach [20]. An exception to this more conservative attitude, is the high grade forms of LN, which are more often segmental in distribution, have a relatively high local recurrence rate and should therefore be treated with surgery (like DCIS) [70]. A strict conservative trend in the management of LN has been favored by some authors to follow the principle of avoiding unnecessary surgery. In this approach the patients are just examined annually and monitored regularly by mammography [71, 72].

LN has usually been managed non-operatively. However, the criteria for the choice of surgical excision or another approach is sill under discussion and different for different clinical centers. There are certain arguments put forward for choosing performing surgical excision after diagnosis with LN such as; significant risk of invasive cancer (controversial reports exist) [9, 73], the limitations of core needle biopsy therefore possible underestimation of the diagnosis [74, 75], existence of suspicious microcalcifications [76, 77] and certain histopathological features (discussed above).
As we mentioned before [56, 57], there are controversial reports on the risk of LCIS diagnosis on contralateral cancers but overall the diagnosis of LN is not a sufficient reason to perform surgery of the ipsilateral or contralateral breast.
Radiation therapy is usually not recommended in the treatment of classic LCIS due to its low radio-sensitivity, but radiotherapy could be an option for the pleomorphic variant which has a higher proliferation rate and high-grade cytology [78, 79].
Systemic treatments are also used for the prevention of progression into ILC. It has previously been shown both pre- and postmenopausal women with LCIS benefits from tamoxifen treatment in terms of prevention of invasive disease [80, 81]. Raloxifene treatment is another option but suggested only for postmenopausal women [81].
In terms of choices as adjuvant therapy, at the moment, there is limited verification existing for adjuvant therapies since surgical excision has not been the primary choice of many surgeons so far. Routine use of tamoxifen has been suggested before [80] however without a supportive argument. Adjuvant radiotherapy has been suggested as

an effective method when performed together with surgery [82].

5.2. Invasive lobular cancer

Surgery is usually the first choice of treatment for the patients with ILC (wide local excision for the area of cancer and surrounding healthy tissue). Since the disease might have spread to multiple areas within the breast, a mastectomy might be offered. ILC regularly metastasizes to the axillary lymph nodes and a sentinel node biopsy is therefore routinely in place [83] generally followed by axillary lymph node dissection only in case of macrometastases to the sentinel node.
Radiation therapy, chemotherapy, endocrine therapy is also applied individually or as a combination therapy [Ref; breastcancer.org].
ILC can metastasize to any distant site, but there is a preference for the gastrointestinal tract, the ovaries and the bone. Systemic treatment in case of distant metastases is as usually based on hormone and HER2 receptor status, preferentially assessed in biopsies of the metastases [84].

6 Conclusion

Lobular breast cancer differs from ductal breast cancer in terms of biology, histology prognosis and response to therapy. Although there are key molecular aberrations associated with this disease (such as loss of E-cadherin expression), investigations regarding the molecular events underlying lobular cancer etiology is limited. This is especially applicable to men where hardly any knowledge on lobular disease has been gained. A better understanding of the molecular pathways governing tumor development and progression are needed to provide new opportunities for therapeutic intervention.

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Chapter 3

Mammary Development and Breast Cancer: The Role of Stem Cells

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Abstract

The mammary gland is a highly regenerative organ that can undergo multiple cycles of proliferation, lactation and involution, a process controlled by stem cells. The last decade much progress has been made in the identification of signaling pathways that function in these stem cells to control self-renewal, lineage commitment and epithelial differentiation in the normal mammary gland. The same signaling pathways that control physiological mammary development and homeostasis are also often found deregulated in breast cancer. Here we provide an overview on the functional and molecular identification of mammary stem cells in the context of both normal breast development and breast cancer. We discuss the contribution of some key signaling pathways with an emphasis on Notch receptor signaling, a cell fate determination pathway often deregulated in breast cancer. A further understanding of the biological roles of the Notch pathway in mammary stem cell behavior and carcinogenesis might be relevant for the development of future therapies.

Introduction

Stem cells in adult tissues are of key importance for physiological tissue renewal and regeneration after injury. Understanding the molecular pathways that govern normal stem cell function may aid in the development of tissue-specific cell replacement therapies whereby stem cells adopt specific cell fates and functions in any desired organ. Although still in its infancy, cell replacement therapy using autologous stem cells holds great promise for overcoming genetic disorders and tissue regeneration after damage. Under pathological conditions such as uncontrolled proliferation and metastases formation in cancer, populations of tumor cells have been identified that are collectively referred to as "tumor-initiating cells (TIC) or cancer stem cells (CSC)" controlling cancer cell maintenance and growth. These cells are thought to harbor many properties of normal stem cells in that they exhibit self-renewing capacity, multipotency and their ability to initiate and sustain neoplastic growth. Increasing evidence indicates that these CSC are responsible for cancer cell maintenance that underlies malignant progression and recurrence after treatment failure.

Breast cancer affects almost 1 in 8 women in the Western world with a total of about one million new cases per year world wide of which 35% will eventually die. Breast cancer is a complex and heterogeneous disease with several histological and molecular manifestations within tumors and between patients. A comprehensive understanding of the etiology of breast cancer is paramount to the identification of novel therapies and improving existing strategies for treatment and prevention of the disease. The mammary gland is a dynamic organ that goes through significant changes during the menstrual cycle, development, pregnancy, lactation, and involution. Normal mammary gland development and homeostasis is a stem cell driven process and key signaling pathways have been identified that control these processes. Mounting evidence indicates that the same genes that control physiological organ development and function are often deregulated in cancer.

Here we provide an overview on the functional and molecular characterization and identification of mammary stem cells in the context of normal breast development and their role in breast cancer. We highlight the key molecular pathways controlling these processes with a special emphasis on the role of Notch receptor signaling, a highly conserved cell fate determination pathway frequently altered in human breast cancer.

Stem Cells

Stem cells can be categorized into two types: pluripotent and multipotent. Pluripotent stem cells are cells that can give rise to the three germ layers, endoderm, mesoderm and ectoderm and propagate by symmetric cell division; i.e. the capacity to give rise to identical daughter cells with identical fates. Examples are embryonic stem cells and embryonic germ cells, which will not be discussed further here. Unlike embryonic stem cells, adult stem cells are multipotent and lineage-restricted and divide by asymmetric cell divisions producing a daughter and a mother at every division with distinct cell fates. Multipotent stem cells are present in many if not all adult tissues and are rare immature

cells characterized by the ability to undergo unlimited self-renewal and differentiate into all cells of a given lineage [1]. Until recently it was assumed that stem cells are quiescent or slow-dividing so-called label retaining cells (LRCs) [2], but that dogma has recently been challenged by the identification of rapidly dividing populations of stem cells in the epidermis and gut where tissue replenishment is a continuous process [3-5]. It has become clear that these organs have both slow and fast dividing stem cell populations that perform different and overlapping functions in regulating tissue homeostasis [6].

The enormous interest in stem cell research over the last decade has lead to the identification of many different markers that allow the identification and purification of cell types with long term repopulating activity in various tissues and organs. So far the most well characterized stem cells are those which form blood, namely Hematopoietic Stem Cells (HSCs) [1]. Currently, there is a good understanding of hierarchical organization of hematopoietic lineage commitment [1]. A similar organization has been observed in other self-renewing tissues such as the intestinal epithelium [7]. Approaches analogous to the ones applied for the hematopoietic system and intestinal stem cells have been employed for the identification of mammary stem cells. Not surprisingly the continuous cell renewal required during each menstrual cycle and for lactation as well as regression in the mammary gland is also controlled by mammary stem cells [8]. The existence of a slow (LRC) and a fast proliferating stem/progenitor populations within the mammary gland has been postulated [9].

Cancer stem cells (CSCs)

Stem cells are obvious targets of accumulating oncogenic mutations due to their longevity and capacity for indefinite proliferation [10]. However, it was only in nineties until John Dick and colleagues established the existence of tumor initiating cells (TICs) or CSCs from myeloid leukemia. By careful immunophenotyping and fractionation of subpopulations of tumor cells, they established that only a fraction of the bulk of the myeloid tumor had the capacity to self-renew in vitro and give rise to transplantable leukemias in vivo [11, 12]. Whereas all cancer cells harbored the initiating mutation, only a fraction was able to initiate and maintain neoplastic growth. From these studies the concept was put forward that leukemias are composed of a hierarchy of undifferentiated immature cells to more differentiated cells with limited potential for self renewal. Soon hereafter colon [13], brain [14], breast [15], pancreas [16], prostate [17], and melanoma [18] CSCs have been postulated. According to this hypothesis, normal stem cells that acquire mutations during tumor evolution continue to exist within tumors and are responsible for the initiation and maintenance of neoplastic growth. TICs retain key stem cell properties such as self-renewal and the capacity to generate progenitor cells, in contrast with the bulk of tumor cells. There is increasing evidence that TICs are enriched in breast cancer patients after conventional treatment, indicating their intrinsic therapeutic resistance [19]. Thus, the first step towards understanding breast carcinogenesis is to identify the pathways that regulate normal breast development and homeostasis. This understanding may lead to insight into the pathways that drive cancer

formation, progression, maintenance and resistance to therapy.

Mammary Gland Development

In mammals the first step in mammary morphogenesis is a thickening of the ventral ectoderm also referred to as the milk or mammary line. This structure gives rise to placodes: the precursor to the mammary bud that will give rise to a ductal branching network rooted within the fat pad and attached to the nipple. Whereas humans only have one pair of placodes that develop into two breasts, mice have 5 pair symmetrically distributed along the rostral-caudal axis between the upper- and hind limbs developing in 10 functional mammary glands. Early mammary gland morphogenesis relies on coordinated signaling between the epithelium and the underlying mesenchyme similar to the development of other epithelial appendages (e.g. limbs, hair follicles). There are important differences however between murine and human mammary gland development. A brief overview of the key steps in mouse mammary gland development is given below and indicated where human development differs significantly.

Mouse mammary gland development

Mouse mammary gland development starts around embryonic day 10.5 (E10.5) and is complete just before birth at E19 day. Just around the time of milk line development Wnt10b expression marks the epithelial and mesenchymal cells destined to form the future mammary gland [20]. Canonical WNT signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis and maintained in the ducts until E15.5. Activation of Wnt signaling induces placode formation and size [21, 22]. Canonical WNT signaling is mediated by the transcription factor Lef1 and epithelial Wnt10b expression is driven by FGF10 produced by mesenchymal cells from the somites which is essential for midline and placode formation [23]. Together with Lef1, Tbx3 has also been shown to be expressed in early mammary gland development. The combination of signaling pathways Tbx3, Fgf and Wnt regulates epithelial-mesenchymal interactions during this time. Both Fgf and Wnt signaling seem to maintain Tbx3 expression while this leads to the expression of Lef1 [24]. It is important to note that due to the spatial distribution of placodes along the rostro-caudal axis, each pair is also exposed to unique signaling cues [25]. During the embryonic development, the mammary gland remains quiescent until E15.5-E16.5 when ductal growth is stimulated by steroid hormones [26]. A combination of steroid and locally acting growth hormones like Insulin-like growth factor [27], estrogen, progesterone, and somatotropin [28] mediate developmental signals and work synergistically in the transmission of these signals to the stromal and epithelial components of the mammary gland [29]. The lumen is generated by apoptosis of central cells in the multilayered epithelium while ducts expand into the fatpads by growth centers with high mitotic rates at the tip of ducts called terminal end buds (TEBs) [30] where stem cells are thought to reside [31].

After birth, ductal growth and branching from TEBs give rise to fully developed mammary

outgrowths between 3-12 weeks of age. Mammary gland goes through changes during pregnancy such as lobulo-alveolar outgrowth (pregnancy), differentiation-secretion (lactation), and apoptotic regression (involution) as part of the normal reproductive cycle [32]. Peptide and steroid hormones together with the interactions of extracellular matrix are responsible of regulating these different phases. Estrogen, progesterone, and prolactin and their cognate receptors are well known regulators required for the successful completion of this developmental cycle [33].

The adult mammary gland is composed of a highly branched system of ducts that terminate in a lobulus. Lobules are composed of alveoli, secretory cells that produce milk. Ducts are composed of two major cell types: an inner layer of luminal epithelial cells and an outer layer of contractile myoepithelial cells which are responsible for contraction in response to oxytocin during lactation [34]. The rapid proliferation and differentiation of mammary gland stem cells at the onset of lactation is necessary to provide secretory activity for milk production [35]. After weaning milk production ceases and the gland involutes [36].

Morphological and functional differences between mouse and human

Mammary development in humans starts as a primary ectodermal outgrowth in 4-6 months old embryo [37]. At this stage, the primary bud contains a central and a peripheral-basal cell population which will give rise to different cell layers [38]. The existence and organization of these distinct cell layers is important in both mice and humans for the correct functioning of the gland and a unique feature of the mammary gland. The epithelial buds start to form from the primary bud in the 21-25 weeks of embryonic age [38] (Figure 1). Breast development differs between individuals of mice at the level of birth. While some individuals have a few branched ducts at that point of development, highly structured ductal tree together with regular lobules as it is observed in adults are also observed [39-41]. After birth, the effect of maternal hormones lessens and the newborn's breast involutes. In females, the ductal tree development and the stromal enlargement continue further only during puberty [42].

Mouse and human mammary gland also differ in terms of morphology. In humans, branching ducts connect to groups of small terminal ductal and alveolar structures which are collectively called terminal ductal lobular units (TDLUs). On the contrary, TDULs are not a feature of mouse mammary epithelial tree. Instead, in the mouse, once the mammary gland is stimulated by hormones, ducts branch and elongate from TEBs (Figure 1). Despite these differences, there is ample evidence supporting overall similar morphology and function including the existence of mammary epithelial stem cells [43].

Developmental patterning and breast cancer

Pathways involved in patterning and morphogenesis during mammary gland development are also implicated in breast cancer formation. Among these are neuregulin3 (NRG3) (an epidermal growth factor receptor (EGFR) ligand [44] involved in placode induction),

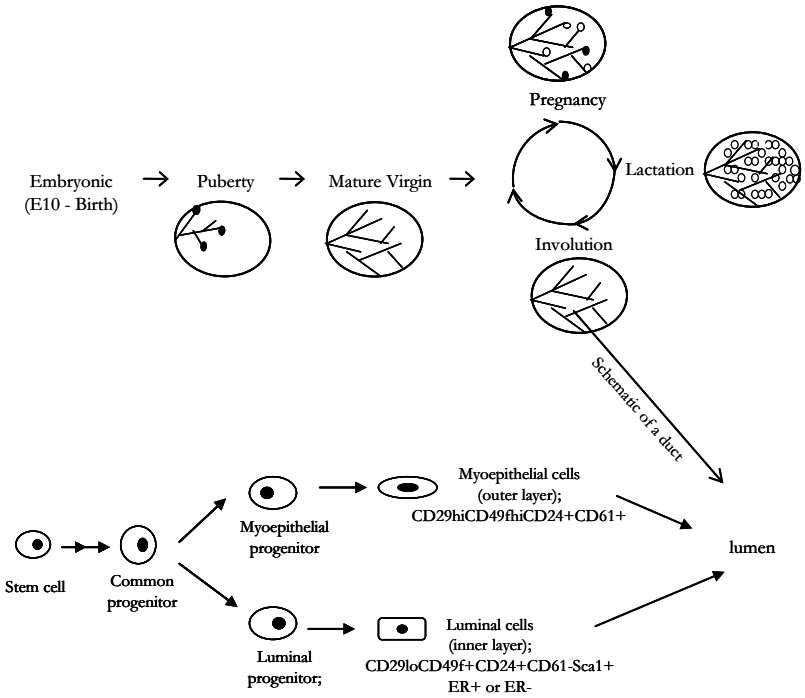

Figure 1. *A schematic representation of mouse mammary gland development and a duct. The closed circular structures appearing at the ductal tips during puberty represent terminal end buds and during pregnancy, also alveolar buds. Mature alveoli are symbolized by the open circles.*

Wnt signalling (essential for midline specification [20]), fibroblast growth factor (FGF) (critical for inductive signaling to the placode [45]) and NOTCH signaling [46] important in luminal cell fate commitment which are all frequently activated/mutated in human breast cancer. Importantly, the same pathways have also been identified by insertion mutagenesis as common proviral insertions in mouse mammary tumor virus (MMTV) induced breast cancer [47] indicating also a causal role for these signaling cascades in breast cancer in mice. Similar insertions by a complete proviral sequence, 95% to 99% homologous to MMTV, were observed also in human breast cancer tissue. This virus was named as Human Mammary Tumor Virus (HMTV) [48]. The percentage of HMTV occurrence is shown to reach up to 42% in breast cancers occurring in Europe, North America [49] and Australia [50] compared to the healthy breast tissues, which do have 1 to 2 % HMTV prevalence. High percentage of insertions seems to play a role in some human breast cancers.

Evidence for Mammary Stem Cells

The postnatal development of the mammary gland makes it possible to surgically remove the rudimentary gland leading to an epithelium free (cleared) fat pad and to study mammary development by transplantation from donor mice. The first functional

evidence for the presence of murine mammary epithelial stem cells comes from transplantation experiments of DeOme [51]. This study showed that small tissue fragments isolated from a randomly selected portion of the mammary gland were capable of regenerating a functional ductal tree upon transplantation into a cleared mammary fat-pad. Moreover, explants taken from a regenerated gland could be serially transplanted to other fat-pads [52]. While these experiments strongly pointed to a long-lived pool of epithelial progenitors, whether these contained "true" stem cells was questioned since transplant ability and ductal outgrowth could only be maintained for up to 7 generations [53-55]. More conclusive evidence for a mammary stem cell was obtained by clonal analysis using insertion mutagenesis with MMTV. These elegant studies demonstrated for the first time that an entire mammary gland composed of luminal and myoepithelial cells was a clonal derivative of a single cell. This "stem cell" was also capable of forming restricted lineages that produced only lobules or only ductal outgrowths without lobules that cannot expand upon impregnation [56]. Accordingly, it appears that mammary epithelial cell populations generated by a single stem cell comprise distinct and multipotent epithelial cells which are able to proliferate. Moreover, MMTV tagged progenitors survived multiple rounds of pregnancy and involution demonstrating that these cell had the capacity for robust proliferation and differentiation and were protected from apoptosis. It is important to note that such a stem-cell population would be exquisitely sensitive to oncogenic transformation.

It took almost another decade for researchers to identify markers that could be used to enrich for this multi-potent mammary stem cell (MaSC) and corroborate these earlier findings [57, 58].

Molecular Characterization of Mammary Stem Cells (MaSC)

Researchers have been using several complementary methods to isolate and study mammary stem/progenitor cells. The isolation of stem cells based on the expression of cell surface markers has been the most commonly used technique. The knowledge obtained in many different systems such as neuronal, hematopoietic and epidermal lineage has been an advantage for the researchers working on the characterization of mammary epithelial stem cells. The following part will provide an overview of the techniques used to identify mammary stem cells.

1- The side population technique; The Side Population or SP is a population of cells that are characterized for their ability to actively exclude vital dyes from being taken up by the cell such as Hoechst and Rhodamine 123 while maintaining other stem cell-like markers [59, 60]. Dye uptake is an active process mediated by ATP-binding cassette family of multi drug resistance proteins e.g. MDR1/P-glycoprotein) and SP staining can be inhibited by drugs such as verapamil. Drug efflux is restricted to normal stem cells and CSC and not observed in differentiated cells. Expression of ABC is closely correlated with stem cells from a wide variety of tissues sources ranging from embryonic stem cells to bone marrow [61]. Mammary gland SP isolated from reduction mammoplasty [62],normal human [63, 64] and mouse mammary gland [65, 66] respectively have

been shown to be enriched by stem/progenitor cells based on their ability to generate lobuloalveolar and ductal outgrowths upon transplantation.

2- Label retention; Label retaining cell (LRC) is another approach to identify stem cells. This approach is based on differential retention of nucleotide analogs (^{3}H-Thymidine (^{3}HTdR) or 5-BromodeoxyUridine (BrdU)) that are incorporated during S-phase between slowly proliferating versus fast proliferating cells. The LRC hypothesis has been very popular for many years particularly for epidermal and intestinal stem cells [2, 67] however recent work has challenged this hypothesis by the isolation of fast-cycling stem cells [6]. LRCs have also been reported in the mammary gland [66, 68, 69]. The undifferentiated nature of LCRs in mammary gland has been identified by the lack of differentiation markers [9]. This population of cells has been shown to divide asymmetrically and is able to repopulate stem cell niches. Although asymmetric cell divisions of stem cells have been proposed before, in a more recent study, Smith et al. tried to answer the question by labeling mice first with ^{3}HTdR and then with BrdU. ^{3}HTdR label was shown to be retained by LRCs in the mammary gland. Following the pulse of BrdU as a second label, most of ^{3}HTdR labeled LRCs were shown to have the BrdU label as well. As a next step, it was shown that these cells were able to undergo asymmetric divisions and pass the newly synthesized BrdU labeled strand to their progeny while retaining the ^{3}HTdR label [69].

3- Hormone receptor status; steroid hormones have a huge impact on mammary development and the majority of breast cancers are estrogen receptor (ER) positive and are an important target for anti-hormonal therapy [70]. In view of this, it is not surprising that researchers have been trying to characterize MaSC with respect to steroid receptor expression. Previously, studies of Clarke et al. demonstrated the existence of steroid receptor positive cells within LRC and SP with full self-renewal and differentiation capacity [71]. On the other hand, more recent studies revealed that both mouse and human MaSCs show a ER, PR and ErbB2 negative phenotype [72]; but yet they respond to ovarian hormone signaling [73].

4-Mammosphere culture; this approach has been used to functionally identify MaSC and TICs based on their ability to form mammospheres in culture that give rise to tumors containing all the differentiated cell types present in the original tumors. [64]. Using this technique, the differentiation capacity (based on the markers specific for different lineages of the mammary gland) and clonality (by retroviral tagging) of cells enriched in the mammospheres was demonstrated. Cells from mammospheres can be serially passaged and retain their multipotency for multiple passages.

5-ALDEFLUOR assay; this is another promising approach to identify stem cells such as hematopoietic and neural stem cells which have high aldehyde dehydrogenase 1 (ALDH1) activity [74]. ALDH1 is a detoxifying enzyme responsible for the oxidation of retinol to retinoic acid and it may have a role in the early differentiation of stem cells [75, 76]. ALDH1$^+$ cells were shown to possess functional and phenotypic characteristics of mammary stem cells even though they seem to be restricted luminal epithelial layer [77]. It has been shown that ALDH1$^+$ cells can survive and proliferate under anchorage

independent conditions and they are capable of self-renewal. Furthermore clonogenic assays demonstrated that these cells do have the ability to give rise to mixed colonies of myoepithelial and luminal cells. ALDH1 expressing cells have been demonstrated in the normal human mammary gland and human breast cancers [78].

6-Cell surface markers; to date, different combinations of cell surface antigens have been used for the isolation of human and mouse mammary epithelial stem/progenitor cells. Although several research groups reached to an agreement on the cell surface expression profiles of stem and certain progenitor cell groups, it is important to keep in mind that the interpretation of the FACS data obtained so far mostly depends on the use of antibodies conjugated to different fluorochromes, antibody titrations and good controls. In the following part, we will review markers used to identify mouse or human mammary stem cells which are summarized in Table 1.

Mammary Gland Stem Cells	Marker
Mouse	CD24, CD29, CD49f, CD61, Sca-1.
Human	ALDH1, c-KIT, CD10, CD24, CD44, CD49f, CD90, CD133, EpCAM, MUC-1

Table 1. *Commonly used surface markers used for mouse and human mammary stem cells*

Mouse Mammary Stem Cells

Defined subsets of mouse mammary epithelial cells have been used to show stem cell capacity by being able to reconstitute mammary glands when transplanted into cleared fat pads [43]. So far, the most useful markers for mouse MaSCs have been CD49 (α6 integrin), CD29 (β1 integrin), CD61 (β3 integrin), CD24, and Sca-1 [79]. Mouse MaSCs could be purified based on $CD24^{+}CD29^{hi}CD49f^{hi}Sca1^{-}$ profile following the removal of stromal and hematopoietic cells [57, 58, 80]. A single genetically tagged cell from this population could give rise to an entire epithelial tree upon transplantation [57]. The self renewal and multi-lineage capacity of these cells was demonstrated by serial transplantations into cleared fat pads leading to mature functional glands with the formation of milk producing alveolar units upon impregnation. Moreover, when these genetically tagged cells were cultured together with wild type cells, they were able to produce chimeric structures [57, 58]. By these experiments, researchers were able to show that the purified cells based on the markers mentioned above did indeed contain stem cells. It is important to note that only a small group of MaSCs is characterized by the $CD24^{+}CD29^{hi}CD49f^{hi}Sca\text{-}1^{-}$ population, others have identified other combinations of markers that indicate stemness (e.g. $CD45^{-}Ter119^{-}CD31^{-}CD49f^{hi}CD24^{med}$. Such populations are not pure since committed progenitors and differentiated cells are also present because these cells express some of these markers as well [58].

Some of the markers used to enrich MaSC populations are interesting due to their regulatory roles. For example high expression levels of integrin α6 (CD49f) and β1

(CD29) both important for adhesion and migration indicates an important role for stemness and microenvironment interactions [81]. It has been previously shown that both α6 and β1 integrin expression decreases during breast cancer progression [82, 83]. Reduced levels of integrins might give rise to reduced interaction of MaSCs with the microenvironment and a failure in the self-renewal and proliferation. Deletion of β1 integrins from Keratin-5 expressing basal cells led to defects in mammary morphogenesis, proliferation and survival of mammary epithelial cells. β1 integrin deficient transplants efficiently regenerated an entire new gland albeit slower than control transplants and were characterized by disorganized ductal outgrowth indicating a lack of regenerative potential in the absence of integrin β1 [84]. A comprehensive review on the role of integrins and extracellular matrix in mammary gland development has recently appeared [85].

Human Mammary Stem Cells (hMaSCs)

Common patterns of X chromosome-inactivation and loss of heterozygosity in adjacent areas of breast epithelium (so-called "Field effect") have provided proof for the clonality of lineages within the normal and neoplastic human mammary gland [86, 87]. Further evidence for a hierarchical model of human breast epithelial development has been obtained by in vitro clonogenic assays where the human mammary stem cells have been shown to reside within the ducts since it was possible to serially passage ductal fragments [64, 88, 89]. The attempts to characterize human mammary stem cells have resulted in identification of a combination of markers such as epithelial cell adhesion molecule (EpCAM), ALDH1 and CD49f [88-92].

Eirew et al. developed a xenotransplantation approach to study the in vivo characteristics of human mammary epithelial cells. A population of cells having $EpCAM^{low}CD49f^{hi}$ profile was sorted by FACS from reduction mammoplasty samples and these cells were subsequently transplanted into immunodeficient mice together with irradiated fibroblasts. The in vivo experiments of Eirew et al. and Lim et al. revealed that $EpCAM^{low}CD49f^{hi}$ population are positioned basally within the mammary gland ducts and posses stem cell characteristics [90, 93]. Even though these two markers do identify different cell types when used alone, together they define a subset of human mammary cells, which have regenerative capacity in a xenograft mouse model. The self-renewal and regenerative capacity of $EpCAM^{low}CD49f^{hi}$ cells was further supported by transplantation into humanized mouse mammary fat pads -Human in Mouse model (HIM)- [93, 94]. In this model, the mouse mammary fat pads are humanized by pre-injection of immortalized human fibroblasts. Using the same method, Ginestier et al. identified another subpopulation of human stem/progenitor cell population expressing high levels of ALDH1, with high engraftment capability into NOD/SCID mice [77]. It should be noted that not only MaSCs but also luminal lineage restricted cells express ALDH1 therefore it is necessary to use a combination of different markers together with ALDH1 such as EPCAM, CD49f, MUC1 and others to obtain the highest enriched fraction of cells with MaSC ability.

Evidence for Tumor Initiating Cells in breast cancer

A large body of evidence has demonstrated the similarities between stem cells and cancer cells. It is well established that stem and progenitor cells are likely targets of genetic mutations necessary for carcinogenesis. The stem cell theory of cancer proposes that there is a small group of cells -cancer stem cells (CSC) or tumor initiating cells (TIC)- within tumors, that fosters tumor initiation and maintenance [95, 96]. The first evidence for TICs was based on studies which showed human acute myeloid leukemia originated from a small proportion of cancer cells [12]. Since then, many groups have investigated and demonstrated the existence of tumor cells capable of self-renewal from hematopoietic to solid cancers by using different techniques [26]. A frequently applied approach has been the transplantation of tumor cells classified by cell surface markers into NOD/SCID mice to investigate the ability of subpopulations of tumor cells to give rise to new tumors. Similar approaches have been used to identify TIC in breast cancer, which will be discussed below. The risk of developing breast cancer is 1 in every 8 women in the western world and it increases with age. This is thought to be related to the accumulation of multiple mutations in cells of the mammary gland over lifetime of women. Damaged stem cells with unlimited potential of proliferation can give rise to delayed cancers such as breast cancer after having a normal phenotype for decades. This is important because once it becomes possible to identify such cells; approaches to eliminate them would decrease the risk of breast cancer incidence and recurrence. The first prospective identification of breast cancer stem cells from human breast cancer specimens reported that within lineage - (tumor cells depleted with expression of normal antigens CD2, CD3, CD10, CD16, CD18, CD31, CD64, and CD140b) population up to 35 % of cells displayed a $CD44^{+}CD24^{-/low}$ marker profile that was able to efficiently generate retransplantable tumors in mice. [15, 97]. Ginestier et al. has showed that $ALDH1^{+}$ phenotype within a number of human breast tumor samples defined a subpopulation of cancer stem cells not overlapping with $EpCAM^{+}CD44^{+}CD24^{-}$ phenotype [62]. When the cells were isolated based on $ALDH1^{+}EpCAM^{+}CD44^{+}CD24^{-}$ phenotype, this population was highly enriched with TIC. Interestingly, a study from Heerma van Voss et al. showed that ALDH1 expression did not differ between BRCA1 (breast cancer 1) mutation carriers (who are at increased risk of developing breast cancer) and non-carriers [78]. Others however have shown a close correlation between Brca1-deficiency and ALDH1 FLUOR positivity in clinical samples and experimental models of Brca1-related cancer [98]. Even though numerous studies [99-102] have proposed the existence of breast TICs, to date the exact nature of these cells remains undefined and far from clinical utility.

Signalling Cues Regulating Mammary Stem Cell Renewal and Breast Cancer

Normal mammary gland development and function is tightly controlled by TGF-β, Wnt, FGF Hedgehog, EGF, Estrogen and NOTCH signaling pathways have been [103]. It has been demonstrated that deregulation of these pathways in the mammary gland can give rise to tumor development. In the following part, we will discuss the role

of these pathways with a focus on NOTCH signaling.

Wnt/β-catenin

Wnt proteins are highly conserved secreted signaling molecules that regulate cell fate throughout metazoan development and homeostasis. Canonical Wnt proteins transduce signals through frizzled (FZD) transmembrane receptors which lead to inactivation of the tumor suppressor protein APC and stabilizes β-catenin which is the intracellular effector of the Wnt pathway [104]. The importance of Wnt pathway activation in breast cancer was demonstrated by identification of Wnt a common insertion site of MMTV [105]. Besides promoting maintenance and proliferation of stem cells, Wnt signaling is also shown to be crucial in pluripotency. The expression of Nanog, a gene known to be associated with pluripotency and self-renewal of stem cells, is suppressed by the Wnt-responsive transcription factor T cell factor 3 (TCF3) [106]. Wnt involvement in pluripotency is further supported by the effect of upregulation of Oct-4, a transcription factor mostly known through its involvement in the inhibition self-renewal of undifferentiated embryonic stem cells [107]. Aberrant activation of the canonical Wnt pathway is also associated with cancer development in early-onset familial adenomatous polyposis (FAP) and late-onset spontaneous forms of colon cancer [108, 109]. Upregulated Wnt activity due to the loss of the Wnt inhibitor SFRP1 (Secreted Frizzled-related protein 1) and high levels of β-catenin is correlated with poor prognosis in breast cancer [110, 111]. Moreover, β-catenin expression has been shown to be associated with survival of mammary epithelial stem cells and more efficient self-renewal [112, 113]. Transplantation of mammary epithelial cells overexpressing constitutively active β-catenin into cleared mammary fat pads gave rise to hyperplasias [112]. These results illustrate that upregulated canonical Wnt signaling increases the mammary stem cell activity in vivo. Conclusive evidence for a role of Wnts in mammary stem cell renewal was recently obtained by the Nusse lab that demonstrated that purified Wnts are sufficient to promote clonogenic growth of mammary stem cells in culture and support their long-term repopulation *in vivo* [114]. It is likely that Wnt induced mitogenic effects in the stem cell compartment is causative to tumor initiation.

Hedgehog

The Hedgehog (Hh) pathway is another highly conserved pathway essential for early embryonic patterning and cell fate determination and in the self-renewal and maintenance adult tissues including mammary gland [115-117]. In mammalian cells, signaling takes place between the three secreted ligands; Sonic Hedgehog (Shh), Desert Hegdehog (Dhh) and Indian Hedgehog (Ihh) and cells that express the transmembrane receptor for Hedgehog ligand Patched-1 (PTCH1) and Patched-2 (PTCH2). In the absence of ligands, Ptch forms a complex with another transmembrane protein called Smoothened (Smo) inhibiting the binding of Smo to Gli: a transcription factor. In the presence of Hh, Patched binds to Hh, enabling Smo to activate Gli proteins (Gli1, Gli2 and Gli3) that activate gene transcription. Deregulated Hh signaling has been shown to

be associated with a range of malignancies. Mutations found in the Hh pathway genes leading to ligand independent activation have been shown to be associated with several malignancies such as medulloblastoma, sarcoma and basal cell carcinoma (BCC) [118, 119]. Overexpression of Hh ligands is frequently observed in gastrointestinal tract and lung carcinomas [120].

The evidence supporting Hh involvement in breast carcinogenesis is increasing. In one of the first studies, Lewis and colleagues showed that heterozygous disruption of Ptch1 and Gli-2, impairs ductal morphogenesis resulting in ductal hyperplasias and dysplasias [121]. Constitutive activation of Smo also leads to aberrant proliferation and ductal dysplasia [122]. The association of Hh pathway with human breast cancer was first demonstrated by Kubo who were able to show the correlation of high levels of Shh, Ptch and Gli1 in human breast cancer tissues and growth inhibition by the Hh inhibitor cyclopamine [123]. Although activating mutations in Hh pathway are common in many cancers, they are not frequently associated with breast cancer [124]. Instead, epigenetic events may be more important in Hh pathway activity in breast cancer as demonstrated by the finding that demethylating and deactylating agents can upregulate Ptch expression in breast cancer cell lines [125]. Finally, Patched polymorphisms have been shown to be associated with the risk of oral contraceptive use on breast cancer risk [126]. This suggests that Hh signaling pathway might an important role in hormone induced development of breast cancer.

Furthermore, in an in vitro study, addition of Shh ligand or overexpression of Gli2 into mammosphere cultures increased primary and secondary mammosphere formation and size, which was reversed by cyclopamine, a specific inhibitor of the pathway. Finally, Hh signaling has been associated with the tumorigenic phenotype of $CD24^{+}Cd44^{-/low}Lin^{-}$ TIC [127]. Altogether, the Hh pathway plays pivotal roles during normal mammary gland development and likely also plays unrecognized roles in breast cancer as well.

Transforming growth factor–beta

The transforming growth factor beta (TGFβ) family of polypeptide growth factors comprise secreted proteins of which there are three isoforms: TGFβ1, TGFβ2, and TGFβ3. TGFβ proteins bind to Type II receptors that heterodimerize to Type I receptors which triggers serine/threonine phosphorylation and activation of receptor SMAD proteins that in turn heterodimerize with other SMAD proteins which enter the nucleus and activate gene-transcription. TGFβ signaling controls virtually all cellular processes during development and in adult tissues including cell proliferation, differentiation, and apoptosis, in species from worms to mammals. Defects in TGFβ signaling occur in many inherited and acquired diseases including cancer [128]. The function of TGFβ in mammary morphogenesis appears to be cell and context dependent. Several groups demonstrated the importance of different local concentrations of TGFβ within ductal epithelium as an inhibitory factor in the ductal growth and lateral branching [129, 130] TGFβ within luminal epithelial cells controls alveolar development [131, 132]. The role of TGFβ in breast cancer is complex as TGFβ has both growth suppressive as well

growth promoting activities. In general TGFβ promotes tumor progression in several ways including suppression of immune responses, stimulation of angiogenesis and promotion of epithelial-to-mesenchymal transition [133, 134].

Estrogens and Progestagens

The steroid hormones estrogen and progesterone have key roles during normal mammary gland physiology from puberty to menopause. Deregulation of this hormonal regulation also markedly influences breast cancer risk forming the basis for anti-hormonal therapies in breast cancer treatment. Although the role of hormones in cancer risk has already been known for a long time it was only recently that two groups reported the underlying mechanism [73], [135]. The thought that TICs would directly respond to hormones is attractive however these cells do not express the receptors for either hormone. It appears that proliferative effect of hormones on breast tissue occurs during the normal reproductive cycle and pregnancy when hormones rise causing significant increases in MaSC numbers. The proliferative effect is indirectly mediated by the response of the Niche (basal and luminal cells) surrounding the stem cells. These produce a Wnt4a signal that stimulates MaSC proliferation. These findings pave the way to understanding why hormones can modulate growth and proliferation of the normal mammary gland and may sustain malignant growth. They also suggest that during such hormonal surges stem cells may be particularly vulnerable for accumulating mutations that lay dormant until later in life when women develop breast cancer. Such breast cancers may ultimately be unresponsive to hormonal therapies if their origin is driven by TICs. In hindsight the relationship between estrogens, MaSC and breast cancer may not be totally unexpected given the interplay between transcriptional regulation of estrogen/progesterone and cyclinD1; a gene frequently upregulated in breast cancer [136, 137]. Mice lacking the cyclinD1 gene have defects in hormone-induced proliferation during pregnancy [138] and are protected from breast cancer [139]. Paradoxically these findings do not explain why pregnancy and breast-feeding in the long term protects against breast cancer in humans [140].

Notch

NOTCH signaling is a short-range cell-cell communication pathway that controls virtually every aspect of metazoan development and cellular responses to maintain tissue homeostasis in adults. NOTCH signaling occurs between transmembrane bound ligands and receptors on adjacent cells and is conserved from flies to mammals. Flies have a single NOTCH receptor and two ligands (Delta and Serrate/Jagged) whereas mammalian cells have four receptors and at least five Delta/Jagged type ligands (Figure 2). NOTCH proteins are single pass type I transmembrane receptors, with an extracellular domain involved in ligand binding, and a cytoplasmic domain involved in signal transduction [141]. During maturation in the trans-Golgi network, NOTCH precursors are first cleaved at Site-1 (S1) by Furin-like convertase producing a heterodimeric type I receptor with the NOTCH extracellular domain (NECD) non-

covalently bound to a transmembrane/intracellular fragment (TMIC). The extracellular domain of NOTCH proteins is composed of 29-36 epidermal growth factor (EGF) like repeats involved in ligand interactions, three cysteïne rich LIN12/NOTCH (LNR) repeats and a heterodimerization domain (HD). The intracellular domain of NOTCH (NICD) contains nuclear localization signals together with a transcriptional activation domain (TAD) and a PEST domain [142]. The canonical NOTCH signaling cascade is regulated by proteolysis. In the absence of ligand mature NOTCH receptors are held into an inactive "proteolysis resistant" state because the Negative Regulatory Region (NRR) composed of the HD domain and the Lin12/NOTCH repeats (LNR) inhibits NOTCH activation. Ligand binding to NOTCH receptors unfolds the NRR permitting cleavage by the metalloproteases ADAM10/Kuzbanian at a site close to the membrane termed site-2 [142] leading to shedding of the NOTCH extracellular domain [142, 143]. Extracellular cleavage of NOTCH triggers the intramembranous cleavage by the multi-subunit protein complex termed γ-secretase containing the aspartyl protease Presenilin. This leads to the release and translocation of NOTCH intracellular domain (NICD) to the nucleus where it interacts with the transcription factor CSL (CBF1 in humans; RBP-J_k in mice) to activate Hes/Hey family genes, which are involved in growth and proliferation, differentiation, and survival [144]. In the absence of NOTCH signaling, CSL inhibits the transcription of target genes. Most if not all NOTCH signaling requires metalloprotease and γ-secretase cleavage to release the NICD and induce transcriptional activation by binding to CSL [145].

Most if not all canonical (CSL-dependent) NOTCH signaling requires γ-secretase cleavage. NOTCH activity is frequently deregulated in human cancer by overexpression or mutation [146]. Mutations are found in the extracellular HD and intracellular PEST domain and induce ligand-independency and increased stability of NOTCH [147]. Mutations that affect the activity of NOTCH are also found in negative regulators such as the ubiquitin ligase Fbw-7 [148, 149]. Given the frequent involvement of Notch in human malignancies, targeting NOTCH cleavage appears an attractive drug target. At the same time this provides challenges for drug development since such drugs would also target physiological NOTCH activation. Several clinical trials are underway that evaluate the efficacy of γ-secretase inhibitors (GSIs) as anti-cancer drugs [150]. While targeting γ-secretase has shown encouraging results in targeting tumors with activated NOTCH it has many pitfalls as well. Among these is the lack of specificity and the mechanism based toxicity caused by attenuating physiological NOTCH function. For example, the gastrointestinal toxicity caused by precocious secretory differentiation of intestinal epithelial cells prevents long-term use because of intestinal stem cell depletion [151-153]. Real et al. showed that such side-effects may be overcome by combination treatment with glucocorticoid and GSIs which suppressed gut toxicity through inhibition of KLF4 [154]. Since γ-secretase has many different substrates, pleiotropic effects are

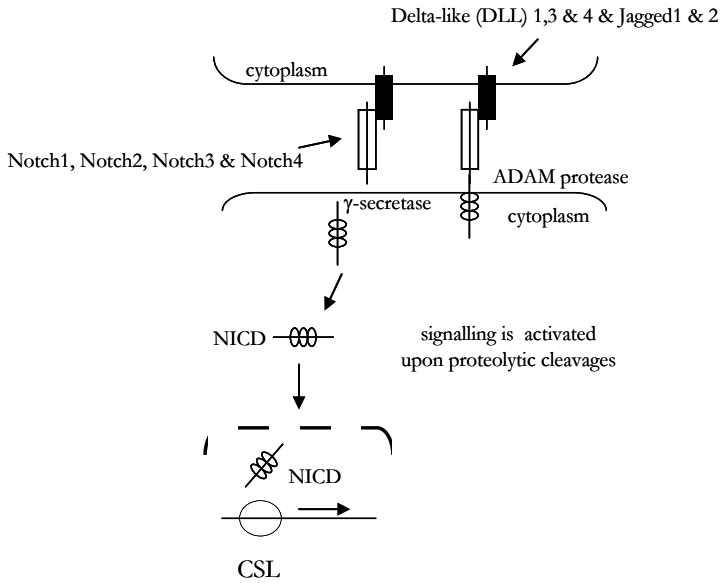

Figure 2. *A schematic of activation of the Notch pathway. Notch signaling is activated by proteolytic cleavages followed by the release and translocation of Notch intracellular domain to the nucleus where it acts on downstream targets by binding the transcription factor CSL to activate Hes/Hey family genes which are involved in cell growth, differentiation, and survival.*

unavoidable when targeting this enzyme, although modulators have been identified that may show selectivity for specific substrates [155]. Despite the fact that GSI have been studied intensely for the past decade, a safe-drug has yet to enter clinical practice [150].

Notch in mammary development

Members of NOTCH family have been shown to play important roles in normal breast development by several studies (Figure 3). In one of the earliest reports, Uyttendaele et al. (1995) demonstrated that normal breast epithelial cells failed to differentiate upon overexpression of a constitutively active form of NOTCH4 in vitro [156]. This was supported by other reports where a constitutively active form of Notch4 was expressed in transgenic mice and lead to a failure in mammary development followed by the progression of mammary tumors [157-159] The increase in mammosphere numbers in mammosphere culture systems upon activation of Notch signaling via an exogenous peptide showed that Notch signaling has a promoting role in self-renewal in human primary mammary epithelial cells [160]. In addition, Notch activation facilitated proliferation and branching morphogenesis which could be inhibited by using Notch antagonists. Altogether, these reports demonstrate the importance of Notch signaling in the regulation of ductal branching in normal development where aberrant Notch signaling seems to disrupt differentiation and causes excessive proliferation.
Notch3, another member of the family, was shown to be upregulated in mammospheres

formed by normal breast tissue [160] suggesting its role in self-renewal. Notch3 has also been shown to be upregulated in breast cancer cells [161]. Causality between deregulated NOTCH signaling and mammary carcinogenesis was exemplified by the identification of NOTCH1 and NOTCH4 loci as common insertions in MMTV induced tumors [162, 163]. The integration of MMTV into the NOTCH4 locus results in a Notch protein lacking most of the extracellular domain creating a ligand-independent protein [164-167]. WAP (whey acidic protein) or MMTV (mouse mammary tumor virus)-driven expression of NOTCH4 in transgenic mice also leads to developmental defects and formation of mammary tumors [158]. The failure in development leads to differentiation defects and hyper-proliferation of immature ductal cells. A possible explanation can be that specified cells within the mammary epithelium govern a proliferating stem cell fate that makes the cells vulnerable to mutational events driving tumorigenesis [158].

The Notch activation is likely to be important for alveolar development since Notch4 overexpression leads to aberrant alveolar growth and lactation in both WAP and MMTV-Int3 female mice [157-159]. This observation was supported by Buono et al. who reported that loss of RBP-J_k and Pofut1, a fucoslytransferases necessary for the activity of Notch proteins, leads to disproportion in differentiation of the luminal and basal cell lineages [168].

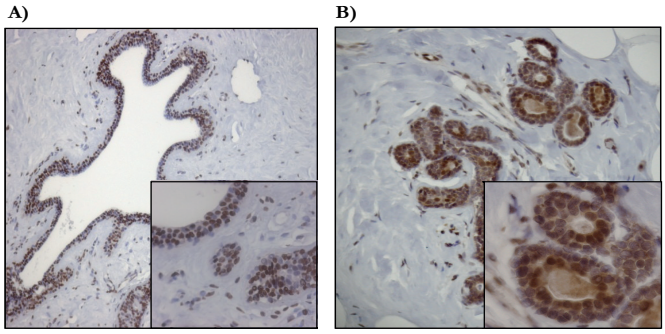

Figure 3. *Notch1 Signaling in Normal Human Breast. We have observed high Notch1 activity in normal human breast epithelium, indicating the activation of this pathway during normal breast development. Normal human breast tissue probed with an antibody against (A) cleaved Notch1 , representing Notch1 activity (10X and 40X magnification), and (B) Hes1 (a downstream target of Notch pathway) (10X and 40X magnification). Similar patterns of expression are evident.*

Notch and Mammary Stem Cells

Dontu et al. were among the first who demonstrated the involvement of NOTCH signaling in mammary stem cell renewal [160]. By utilizing the mammosphere system, they were able to show that human mammary stem/progenitor cells formed an increasing number of mammospheres when NOTCH signaling was activated, and that sphere formation was inhibited when NOTCH signaling was blocked. In the same study, they demonstrated the role of NOTCH4 in myoepithelial lineage commitment and branching morphogenesis of human mammary stem/progenitor cells in three

dimensional matrigel cultures. More genetic approaches have also been used to block the NOTCH pathway, such as the targeted disruption of RBP-jk in the mouse mammary gland [168]. Even though no aberrant changes were observed in virgin animals, alveolar cell maintenance and basal cell proliferation increased dramatically by the loss of RBP-jk upon pregnancy. Thus NOTCH appears to be regulating alveolar development during pregnancy by controlling the luminal cell fate since the luminal cells fail to differentiate upon loss of NOTCH signaling. The role of NOTCH signaling in luminal cell fate commitment was supported later by Bouras [169], who demonstrated high Notch1 activity in luminal progenitor cells in vivo. Moreover, constitutive activation of NOTCH1 in $CD29^{hi}CD24^{+}$ cells was shown to stimulate luminal cell fate commitment leading to excessive proliferation and eventually tumorigenesis. Conversely, knockdown of RBP-Jk led to an increase in number, size and clonogenic capacity of $CD29^{hi}CD24^{+}$ mammary epithelial cells. Notch pathway inhibition induced aberrant luminal cell differentiation and expansion of basal cells. In contrast, NOTCH4 was shown to be down-regulated in luminal restricted stem/progenitor cells whereas NOTCH3, was shown to be upregulated [169]. Functional studies supported this further where NOTCH3 signaling was blocked and this retarded the luminal cell fate commitment where it stimulated myoepithelial cell differentiation. This was further confirmed by Rouf et al.'s work, where they performed a transcriptome analysis and showed that upregulation of NOTCH3 and down-regulation of NOTCH4 characterized the luminal cell lineage [91].

Notch in Mammary Carcinogenesis

MMTV insertions in the NOTCH1 locus were first identified in MMTV-Neu mammary tumors [162] as a collaborating oncogene in ErbB2/Neu driven mammary tumorigenesis. NOTCH1 was shown to be rearranged in 2 out of 24 MMTV-Neu mammary tumors investigated. These insertions caused expression of constitutive active truncated NOTCH1 proteins in a similar manner to that observed in the case of NOTCH4. Wnt and NOTCH signaling also collaborate in the transformation of human primary mammary epithelial cells (HMECs) [170]. In this study, ectopic expression of Wnt1, induced overexpression of NOTCH ligands (Dll1, Dll3, and Dll4). Further, it induced Notch receptor cleavage which leads to NOTCH activity resulting in mammary epithelial transformation. HMECs with constitutive activation of NOTCH1 detached from culture plates and began to form spherical structures. However these spheres failed to proliferate, indicating that NOTCH1 activation alone was not sufficient to induce transformation of these cells. Interestingly, Notch1 activation in 3 dimensional cultures of MCF-10A, an immortalized human mammary epithelial cell line, yields heterogeneous phenotypes such as large and hyper-proliferative structures or small and growth arrested structures [171]. Based on these results, it is postulated that different phenotypes are related with dose and context dependence of Notch pathway [172]. There is increasing evidence that activation of NOTCH signaling is a frequent event in human breast cancers. In one of the earliest studies, immunohistochemical analysis of

NOTCH1 was observed in high percentage (~57%) of breast cancers in concert with high Ha-RAS expression [173]. Further in vitro experiments revealed that oncogenic Ha-RAS acts at a post-transcriptional level to increase NOTCH1 activity and that Ha-RAS induced transformation requires NOTCH1 activation. High NOTCH1 levels have been correlated mainly with poorly differentiated breast tumors suggesting a possible defect in cell fate decisions, whereas NOTCH2 expression is inversely correlated to poor prognosis [174]. These data support the notion that different members of the NOTCH family might have different roles depending on cellular context. Analysis of several human breast cancer cases showed high JAG1 levels besides high NOTCH1 and NOTCH3 expression in association to poor overall survival [100, 161, 175]. These findings are in line with research demonstrating the tumor-promoting role of NOTCH1 and NOTCH3 in mice [100, 176]. Similarly, Pece et al. showed that loss of NUMB expression (a negative regulator of the NOTCH pathway) is commonly observed in primary human breast cancer [177].

Notch as a prognostic tool and a therapeutic drug target

To date, it is clear that the NOTCH pathway plays a crucial role in breast cancer development. Therefore, targeting Notch activity in cancer may provide a bona fide approach for the development of novel therapeutic intervention strategies. Strategies include: (i) preventing ligand-receptor interaction using soluble ligand [178, 179], (ii) ectodomain blocking antibodies [180-183] (iii) targeting metalloprotease cleaveage [143, 184] and (iv) targeting γ-secretase cleavage [173]. Crosstalk between Notch signaling and other pathways frequently altered in breast cancer are exploited to sensitize breast cancers to common cancer therapeutics drugs by combined Notch inhibition [185, 186]. Finally, approaches to block transcriptional activation have also been successful and may be implemented for future treatment regimes [187].

Inhibition of gamma-secretase activity has been so far the most well developed approach to prevent the activation of NOTCH signaling. Gamma secretase inhibitors (GSIs) have been heavily investigated since the gamma secretase complex also cleaves Aβ peptide which plays an important role in Alzheimer's disease [188]. These inhibitors can also block the NOTCH pathway, generating increasing interest for their potential usage as new cancer therapeutics. In case of breast cancer treatment, GSIs seem to have potential in combination with other therapies for individualized therapy. The optimal use of NOTCH inhibitors was demonstrated to be dependent on ER status of the tumor. It has been suggested that GSIs might be more efficient in combination with chemotherapy for the treatment of ERα/PR negative tumors [189] which do not overexpress Her2/Neu. Accordingly, another study showed that inhibition of Her2 overexpression by trastuzumab or tyrosine kinase inhibitors (TKI) increases NOTCH1 activity, which then sensitized breast cancer cell lines [185] for GSI treatment.

Expression patterns of NOTCH pathway genes are also likely to have a prognostic relevance. Recent studies stressed the importance of the detection of NOTCH expression as a potential prognostic marker in breast cancer. Yao et al. demonstrated the

correlation of the expression of NOTCH, NOTCH4 and Jag1 with known prognostic factors such as ERα status, tumor grade, Ki67, lymphovascular invasion, lymph node status and tumor size [190].

Conclusions

The existence of MaSCs was already demonstrated more than 50 years ago but we are only beginning to understand the identity of these cells, the signaling pathways that regulate their homeostasis and their role in diseases such as breast cancer. Currently, there are a number of important questions remaining to be answered; what are the functional differences between human and murine stem cells in the mammary gland? Where do these stem cells reside within their organs and in what micro-environment (niche)? This knowledge is needed to improve our understanding of breast stem cells and their role in cancer formation and progression. Notch signaling has emerged as an important cell fate determinant in mammary gland morphogenesis where it plays seemingly opposite roles in cell renewal and differentiation. Notch is also frequently deregulated in breast cancer and in some cases expression of Notch or its ligands has shown prognostic significance. The γ-secretase inhibitors are emerging as potent drugs that attenuate Notch signaling and are currently being evaluated in clinical trials including those with breast cancer. The coming years will reveal if and how breast cancer patients may benefit from treatments with Notch inhibition.

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Chapter 4

NOTCH Inhibition Induces Growth Arrest in Lobular Breast Cancer

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Manuscript

Abstract

The lifetime risk of developing breast cancer is about one in eight for women in the western world and one of the leading causes of cancer-related death in women. 10%–15% of breast cancer patients are diagnosed with invasive lobular carcinoma (ILC); the second most prevalent type of breast cancer. There is a strong need for a better understanding of ILC to improve treatment. Here, we address the role of NOTCH signaling in lobular breast cancer. We observed a high frequency of NOTCH pathway activity in human ILC patient material. Importantly, *in vitro* inhibition of NOTCH signaling using γ-secretase inhibitors (GSI) led to proliferative arrest in both mouse and human ILC like cell lines. Furthermore GSI treatment in a mouse allograft model for ILC inhibited NOTCH signaling and retarded tumor growth. Our data reveals NOTCH signaling as an important pathway for the growth and maintenance of ILC, making it an attractive candidate for individualized treatment of these patients.

Introduction

Breast cancer is the most frequently occurring malignancy among women in the western world, affecting almost 1 in 8 women with approximately one million new cases per year world wide, of which 35% will eventually die. Breast cancer is a complex disease with several histological and molecular manifestations within tumors and between patients [1]. Invasive ductal carcinoma (IDC) is the most prevalent form of breast cancer, whereas 10%–15% of patients present with invasive lobular carcinoma (ILC), and some other minor subtypes [2, 3]. Lobular carcinomas differ from ductal carcinomas in terms of biology, histology and response to therapy. ILC is often multifocal and bilateral [4-6]. Histologically, ILC manifests as small and round shaped non-cohesive cells which are highly dispersed and locally invasive, making diagnosis with physical examination and mammography difficult [7]. The majority of ILCs are more frequently hormone receptor positive than IDC [8]. Although ILCs initially often respond to endocrine therapy, they become resistant once the hormone receptor expression is lost, which often occur in tumors with high histological grade [9]. Unlike IDC, ILC more frequently shows low/absent EGFR and HER-2 expression [3], making them less suited for anti-EGFR family monoclonal antibody or small molecule based therapies (e.g. CetuxiMab, Lapatinib, Herceptin) [10, 11]. Overall, ILC has a worse prognosis compared to IDC [8]. This reflects the low success rate of current treatment strategies against ILC. Although there are key molecular aberrations associated with ILC (such as loss of E-cadherin expression), investigations regarding the molecular events underlying lobular cancer etiology is limited [12, 13]. A better understanding of the molecular pathways governing ILC development and progression are needed to provide new opportunities for therapeutic intervention.

The NOTCH signaling pathway is a cell fate specification and self-renewal pathway important for normal breast development and cancer [14]. In mammals the NOTCH family consists of 4 transmembrane receptors and 5 membrane bound ligands. Ligand-receptor interactions between adjacent cells induce a series of proteolytic cleavages that result in the release of the NOTCH intracellular domain (NICD) from the plasma membrane which translocates to the nucleus to initiate gene transcription. This intramembranous cleavage is performed by γ-secretase -the Presenilin containing enzyme complex- which is required for most NOTCH activity[15].

Deregulated expression of NOTCH ligands and receptors is common in human cancers [16]. In about 50 % of T cell acute lymphocytic leukaemias (T-ALL) activating mutations in NOTCH1 are present, and inactivating mutations in the ubiquitin ligase FBW7/SEL10/CDC4 also increase NOTCH activity in T-ALL [17-19]. Aberrant NOTCH signalling is also implicated in the development of breast cancer. For example, *Notch1* and *Notch4* loci have been identified as common insertion sites of the Mouse Mammary Tumour Virus (MMTV), which induces mammary cancers in mice [20, 21]. Forced expression of activated NOTCH4 or NOTCH1 in mammary epithelial cells also leads to transformation and rapid development of poorly differentiated adenocarcinoma [22, 23].

In human breast cancer there is mounting evidence for an important role of deregulated NOTCH signaling. For example, in early breast lesions such as Ductal Carcinoma in Situ (DCIS), NOTCH1 signaling is active and associated with breast cancer recurrence [24]. Also in advanced breast cancer NOTCH1 receptor and JAGGED1, a NOTCH ligand, expression are associated with basal phenotypes and poor prognosis [25, 26]. Furthermore, NOTCH4 can transform primary mammary epithelial cells and influences the ability of mammary progenitors to form mammospheres in vitro [27], a property associated with the ability to form transplantable tumors in mice [28]. Altogether these studies suggest that NOTCH4 is more important in maintaining undifferentiated progenitors whereas NOTCH1 and NOTCH3 are implicated in secretory or luminal differentiation [29-31].

Despite the amount of knowledge we have on the role of NOTCH signaling in the development and progression of ductal breast cancer, little is known about the importance of this pathway in ILC. Currently several clinical trials are ongoing for invasive ductal cancer using NOTCH inhibitors (γ-secretase inhibitors or GSI). Whether such a therapy would also work in the more aggressive therapy resistant lobular cancers is not known.

In this study, we report frequent NOTCH activation in human lobular breast cancer and we demonstrate therapeutic feasibility of using NOTCH inhibitors in a mouse model for ILC.

Materials and Methods

Tissue samples and tissue micro arrays

Formaldehyde-fixed paraffin embedded breast cancer tissue blocks of 240 cases were collected from the archives of Department of Pathology of University Medical Centre of Utrecht, to prepare tissue micro arrays (TMAs). For TMA construction, representative areas containing morphologically defined tumor tissue were identified on haematoxylin-eosin stained reference slides by an expert pathologist (PJvD). The tissue micro array was constructed by transferring tissue cylinders of 4-5 mm from the representative tumour area of each donor block to the recipient block using a tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA). Serial sections of 4 μm were cut from this TMA and transferred on Superfrost+ slides (Menzel and Glaeser, Germany) for immunohistochemistry experiments.

Immunohistochemistry

Immunohistochemistry was performed on conventional 4 μm slides by using the following markers; activated NOTCH1 Val 1774 antibody (N1ICD antibody) (Cell Signaling Technologies, Danvers, MA, USA), activated NOTCH1 antibody (Abcam, Cambridge, UK), HES1 antibody (Millipore, Billerica, MA, USA), Ki67 (Thermo Scientific, USA) and cleaved Caspase3 (Cell Signaling Technologies, Danvers, MA, USA). Briefly, tissue sections were deparaffinised and rehydrated. Endogenous peroxidase was blocked with methanol / 0.3% hydrogen peroxidase by immersing the slides in

the solution for 15 minutes. Slides were heated in Citrate or EDTA buffer depending on the antibody used. Unspecific binding sites were blocked with a 1:50 normal goat serum in PBS (pH: 7.4) containing 0.1% sodium azide and 1% BSA. Polyclonal rabbit activated NOTCH1 antibody (Cell Signaling) was diluted 1:100, polyclonal rabbit activated NOTCH1 antibody (Abcam) 1:500, HES1 antibody (Millipore) was diluted 1:600 in PBS/1%BSA, Ki67 was diluted 1:2000 and cleaved Caspase3 antibody (Cell Signaling) was diluted 1:1000. Tissue slides were incubated at 4 °C overnight in a humidified chamber, and then incubated with a HRP conjugated secondary antibody followed by diaminobenzidin (10 min), counterstaining with hematoxylin, dehydration, and coverslipping. Appropriate positive and negative controls were used throughout.
For PAS staining; tissue sections were first deparaffinised and rehydrated and then treated with 1% Periodic acid for 5 minutes. After this step, the sections were washed with demi water and treated with Schiff 's reagent for 15 minutes. Following this step, sections were washed with demi water and rinsed with running tap water and then counterstained with hematoxylin, dehydrated, and coverslipped.

Scoring

For active NOTCH1 staining, the percentage of positively stained nuclei was estimated. N1ICD staining in more than 10% of cells was considered as positive. For HES1 staining, percentage and the intensity of positively stained nuclei (scored semi-quantitatively 0-3) were estimated by an experienced pathologist (PJvD). A standard light microscope was used for the evaluation of staining, at 20-40X final magnification. Pictures were taken with a Leica digital camera DMX1200 through 10X, 20X and 40X objectives. The TMA tissue cores were not scored if they were missing.

Cell lines and culture conditions

Luciferase expressing mouse ILC breast cancer cell line KEP1.11 and WEP3, were derived from spontaneous tumours from K14cre; *Ecad*$^{F/F}$;*p53*$^{F/F}$ and WAPcre; *Ecad*$^{F/F}$;*p53*$^{F/F}$ mouse breast cancer models which have been described before [32]. Murine ILC lines were grown in DMEM/F-12 supplemented with 10% fetal calf serum, 100IU/ml PeniStrep, 5ng/ml Insulin And 5ng/ml EGF (all from Invitrogen Life Technologies). Human breast cancer cell lines used for DNA sequencing and functional analysis (ZR-75-30 and MDA-MB-453) were a gift from dr. J. W. Martens (Erasmus MC, Rotterdam, the Netherlands) and were grown in standard culture flasks in DMEM medium supplemented with 10% fetal calf serum, PeniStrep in a humidified incubator at 37 °C at an atmospheric pressure in 5% (v/v) CO_2. T-ALL cells lines (JURKAT and DND41) were cultured in the same medium and under the same conditions.

Western blot

The human and mouse breast cancer cell lines were treated with 1 μM of the GSI, DBZ, or vehicle DMSO for overnight in a humidified incubator at 37 °C at atmospheric pressure in 5% (v/v) CO_2. Cells were then lysed in lysis buffer (1% Triton, 50 mM Tris pH=7.6, 150 mM NaCl, 1X Protease inhibitor completed with H_2O) for 30 min on ice.

Lysates were spun down for 15 min at 14000 rpm at 4 °C. For western blot analysis, equal number amount of protein was added to 6x Laemmli buffer. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes. Protein detection was performed with subsequent primary antibodies: activated NOTCH1 Val 1774 antibody (N1ICD antibody) (Cell Signaling Technologies, Danvers, MA, USA), activated NOTCH1 antibody (Abcam, Cambridge, UK), HES1 antibody (Millipore, Billerica, MA, USA) and ACTIN antibody (MP Biomedicals, California, USA) Secondary antibodies used were anti mouse (DAKO, Denmark) and rabbit (Jackson Immuno Research, Baltimore, USA) IgG HRP linked antibodies and detected using ECL (Amersham Biosciences, UK).

Proliferation and cell cycle assay

The effect of GSI treatment on the proliferation of human and mouse breast cancer cell lines was investigated by using a FITC BrdU Flow Kit (according to the manufacturer's instructions, BD Biosciences). Flow cytometric measurements were performed using a FACSCalibur (BD Biosciences) and data were analyzed by CellQuest software (BD Biosciences). The cell lines were treated with 1 μM of the GSI, DBZ, or vehicle DMSO, which was refreshed daily, for 4-7 days in a humidified incubator at 37 °C at atmospheric pressure in 5% (v/v) CO_2.

Apoptosis assay

Apoptosis of the GSI treated and control cells were measured by using the Annexin V-FITC Apoptosis Detection Kit (Bender MedSystems, San Diego, USA). As a positive control, the cells were treated with Staurosporine (1 μM, for 24 hrs).The staining was carried out according to the manufacturer's instructions. Flow cytometry measurements were performed using FACSCalibur and the results were analyzed by CellQuest software.

Immunofluoresence

To address further if GSI induces apoptosis in human and breast cancer cell lines, immunofluoresence staining against cleaved Caspase-3 was performed. Cells were plated on sterilized glass coverslips placed in 12-well plates. After treatment with 1 μM of the GSI, DBZ, or vehicle DMSO, which was refreshed daily, cells were incubated for 4 days in a humidified incubator at 37 °C at atmospheric pressure in 5% (v/v) CO_2. At the end of the experiment, cells were fixed and permeabilized with ice cold 100% Methanol for 10 minutes. Fixed cells were blocked with 2% BSA for 1 hr at RT and incubated with a primary antibody raised against cleaved Caspase-3 (Cell Signaling, 1:50) overnight at 4°C. Next day, the cells were washed and incubated with a secondary antibody Alexa Fluor 555 (Invitrogen, 1:500) for 1hr at RT. Before the final washing step, DAPI staining was performed for visualization of nuclei. The imaging was performed by Leica DMI4000b fluorescence microscope.

NOTCH inhibition in an orthotopic model for ILC

For *in vivo* experiments, we used mouse ILC cell lines (KEP1.11 cell line) isolated from

an established mouse model of ILC, based on conditional disruption of *Cdh1* and *Trp53* [33]. For our experiment, KEP1.11 mouse breast cancer cells were orthotopically transplanted into the cleared mammary fat pads of female nude mice (n=13 for each group). After this, mice were examined regularly for tumor size with a digital caliper. Once tumors were palpable (~50 mm^3), bioluminescent imaging and caliper measurements were performed. To calculate the effect of GSI on tumor volume, we have excluded the tumors which are smaller than 20 mm^3 in control and drug treated groups. This approach provided a reproducibility measure.

Vehicle only or drug administration was performed using Alzet Osmotic Pumps (DURECT Corporation, Canada). Control group (n=13) was given only vehicle and drug group was given the GSI, DBZ, (10 micromole / kg) daily/ for 10 days, as described previously [34]. At the end of the treatment period, the mice were euthanatized and the tissues were harvested for further analysis. Mice were housed in pathogen-free conditions at Animal Facility of University of Utrecht in compliance with IACUC regulations. Experimental protocols were conducted with permission from the local animal experimental committees (DEC) in compliance with the Dutch law on animal experimentation.

Statistical analysis

The Chi-square test was used to evaluate correlations between protein expression and histological type of the tumor. For statistical analyses of proliferation assay, cell cycle assay, two way ANOVA test was applied and for tumor volume measurements and goblet cell count in the gut, the Mann-Whitney U test was used to calculate the significance levels of differences between the control and GSI treated cells/groups.

Results

High NOTCH1 activation correlates with lobular breast carcinomas

To investigate NOTCH1 activation in human breast cancer specimens, we screened a TMA containing 240 human breast cancer specimens of which 125 IDC and 115 ILC using an antibody detecting the activated cleaved form of NOTCH1 (Val1744 cleaved NOTCH1). In normal breast tissue, we observed N1ICD in the luminal epithelial cells in line known NOTCH1 expression and activity patterns (Figure 1A). A similar observation was made for the common downstream target of NOTCH pathway, HES1 (Figure 1B). In breast cancer specimens, high N1ICD and HES1 expression was observed in a significant proportion of ILC cases compared to IDC (Figure 1C, D and Table 1) ($p<0.001$). Moreover, ILC cases with high N1ICD expression were also mostly HES1 positive ($p=0.002$) (Table 2).

NOTCH inhibition causes proliferative arrest in lobular breast cancer cell lines

To further study the phenotypic consequences of NOTCH activation in ILC, we used two human and two mouse breast cancer cell lines with similar characteristics to ILC.

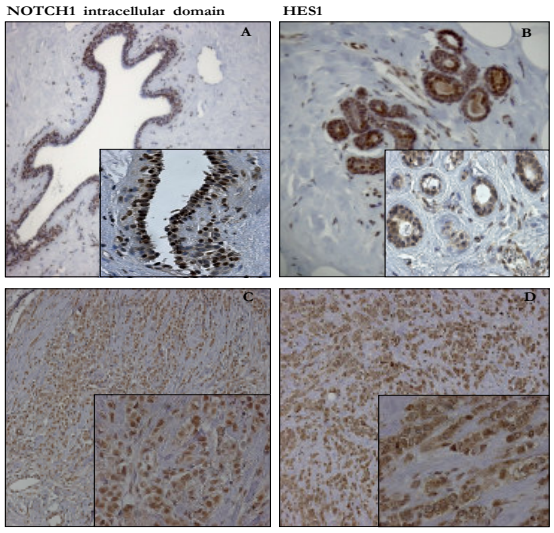

Figure 1. *Immunostaining of normal human breast and breast cancer tissue for NOTCH1 ICD and HES1 A) A representative immunohistochemistry picture of N1ICD staining on normal breast tissue B) HES1 staining in normal breast tissue C) N1ICD staining in a lobular breast cancer specimen D) HES1 staining a lobular breast cancer specimen. Pictures were taken with a Leica digital camera DMX1200 through 10 X and 40 X objectives.*

		IDC	ILC	
N1ICD	-	48 (%53)	19 (%19)	p <0.001
	+	42 (%47)	83 (%81)	
HES1	-	67 (%68)	30 (%32)	p<0.001
	+	31 (%32)	64 (%68)	

Table 1. *Expression of NOTCH1 ICD and HES1 in human invasive ductal (IDC) and lobular invasive carcinomas (ILC) * The TMA tissue cores were not scored if they were missing. There were 35 missing cores for IDC and 13 for ILC cases.*

First, we confirmed NOTCH1 and HES1 expression in these cell lines by analyzing control and/or GSI treated cell lysates by immunoblotting for cleaved and activated NOTCH1 and HES1 (Figure 2A). All four cell lines showed active N1ICD indicative of NOTCH1 cleavage and activity which was confirmed using HES1 immunoblotting. We observed that N1ICD formation and HES1 expression were lost upon GSI treatment. Therefore the NOTCH pathway is active in cell lines with characteristics of ILC.

Next, we asked if the growth and proliferation of the human and mouse cell lines are affected by NOTCH inhibition. The T-ALL cell line JURKAT harbors mutations in both NOTCH1 as well as in FBW7 and is known to be resistant to GSI [18]. As expected JURKAT cells showed no change in BrdU incorporation when treated with GSI. The GSI sensitive DND41 T-ALL carrying activating NOTCH1 mutations responded to GSI inhibition by a reduction in DNA synthesis as measured by S-phase labeling using 5-bromo-2-deoxyuridine (BrdU) incorporation. (Figure 2B).

		N1ICD	
		-	+
HES1	-	39	41
	+	22	63

Table 2. *Correlation of activated NOTCH1 (N1ICD) and HES1 expression in human ILC. p=0.002 * The missing TMA tissue cores for each staining were excluded.*

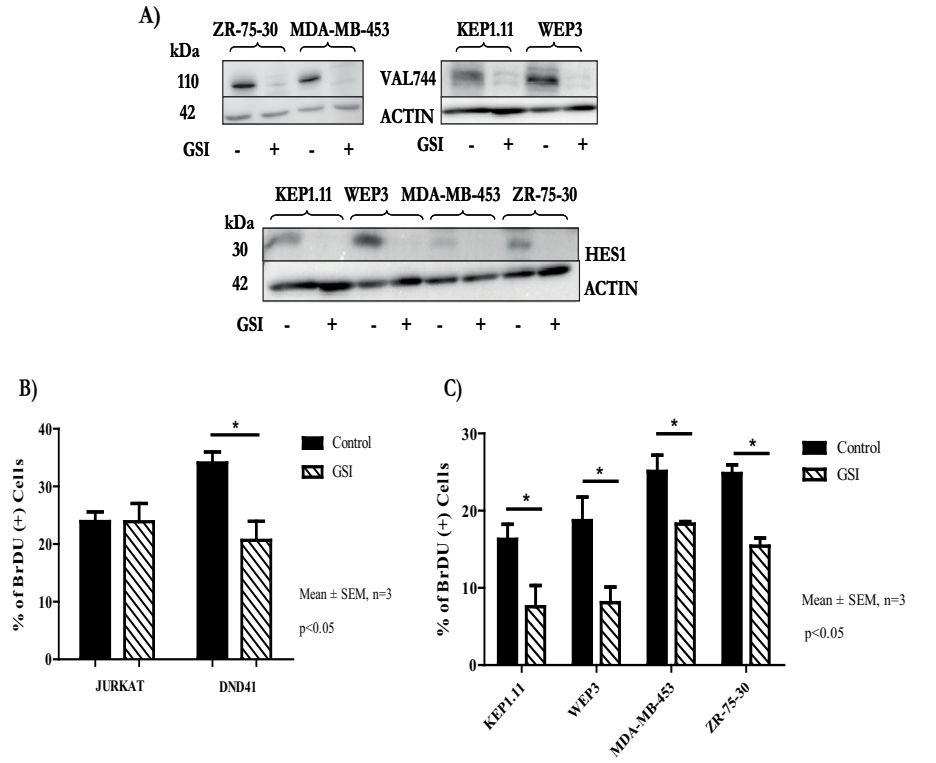

Figure 2. *Mouse and human breast cancer cell lines are sensitive to GSI treatment. In A,B and C, cells were treated wither with DMSO or GSI A) Lysates of human (ZR-75-30, MDA-MB-453) and mouse (KEP1.11, WEP3) breast cancer cell lines were blotted for N1ICD expression with Val1744 (upper panel) or for HES1 expression (lower panel). Total protein levels for ACTIN were determined as loading control. B) The effect of NOTCH inhibition was monitored in T-ALL cell lines JURKAT and DND41 by S-phase labeling using 5-bromo-2-deoxyuridine (BrdU) incorporation and following FACS measurement. C) Effect of GSI on the proliferation of human and mouse breast cancer cell lines.*

Using these conditions, we exposed growing cultures of mouse and human ILC like cell lines to GSI for the duration of 4-7 days and analyzed them for BrdU incorporation. All cell lines showed reduced proliferation ranging from 30% to 50% when cultured in the presence of GSI (Figure 2C). To address if the reduced proliferation caused by NOTCH inhibition was accompanied by increased cell loss or apoptosis, we stained the cells with PI and apoptotic marker Annexin-V. In all cell lines we observed a low level

of cell loss (1-3%) by PI-Annexin-V staining (data not shown).

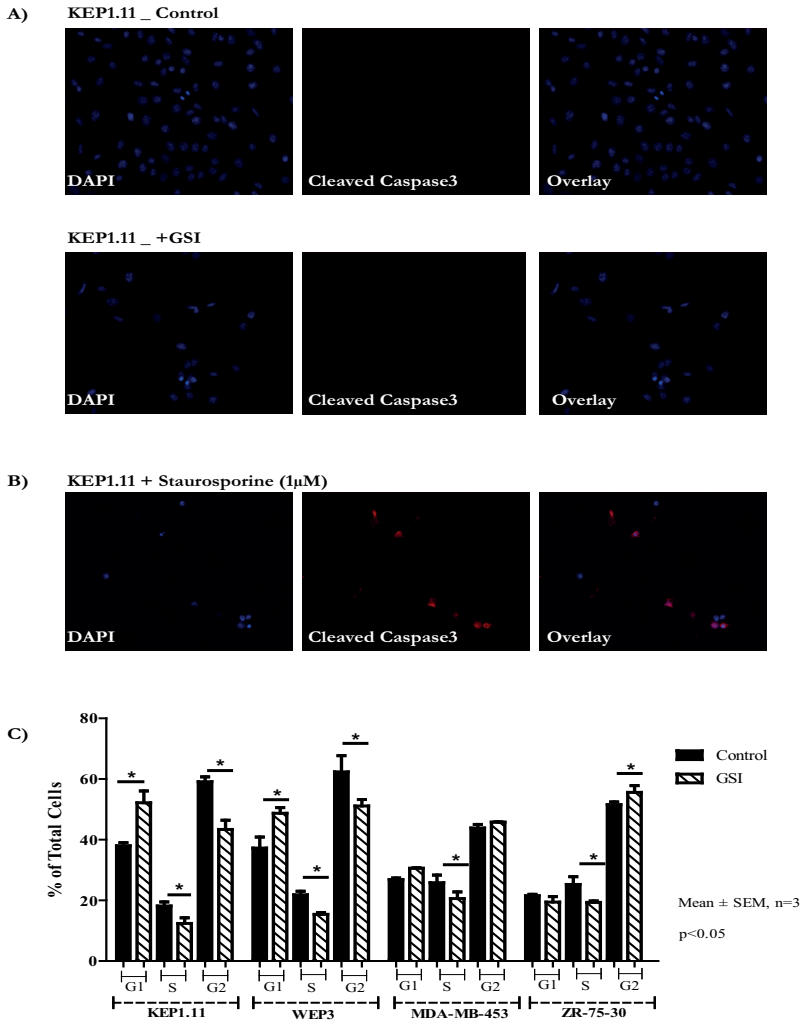

Figure 3. *GSI treatment does not induce apoptosis in mouse and human breast cancer cell lines. A) To monitor the effect of NOTCH inhibition on apoptosis, control and GSI treated cells (After 7 days) were stained against cleaved Caspase3, a commonly used apoptosis marker. In all cell lines we observed very low level or no of cell loss. GSI treatment did not increase apoptosis in any of the cell lines tested. B) To test whether mouse ILC cell lines were intrinsically resistant to apoptosis, we exposed the cells to the apoptosis inducer Staurosporine. We observed massive apoptosis induced by Staurosporine in KEP1.11 cells indicating the apoptotic machinery is intact. (The images are representative for other cell lines.) C) Human and mouse breast cancer cell lines were labeled with 7-AAD (7-amino-actinomycin, a total DNA stain) and BrdU to distinguish S-phase G1 diploid (2n) and G2 tetraploid (4n) populations. FACS measurements were analyzed by FCS Express 4 Software. In both*

human ZR-75-30 and MDA-MB-453 cells GSI treatment induced a slight but significant reduction in S-phase labeling, but little accumulation in G1 or G2 phases. In both mouse ILC lines we observed a clear increase in G1 arrested cells with a concomitant reduction in S and G2 phase in GSI treated cells versus controls.

We did perform immunofluoroscence staining against cleaved Caspase3 to visualize any apoptosis occurring and tested whether mouse ILC cell lines were intrinsically resistant to apoptosis by exposing them to the apoptotic inducer Staurosporine. GSI treatment did not increase this cell loss in any of the cell lines tested during 7 days of treatment (Figure 3A and B). We observed significant apoptosis induced by Staurosporine in murine KEP1.11 ILC cells indicating the apoptotic machinery is intact (Figure 3B).
Cell cycle arrest and redistribution may also underlie decreased S-phase labeling. To address this we pulse labeled cells with 7-AAD (7-amino-actinomycin D) a total DNA stain and BrdU to distinguish S-phase G1 diploid (2n) and G2 tetraploid (4n) populations. In both ZR-75-30 and MDA-MB-453 cells (human breast cancer cell lines) GSI treatment induced a slight but significant reduction in S-phase labeling, but little accumulation in G1 or G2 phases. In both mouse ILC lines we observed a clear increase in G1 arrested cells with a concomitant reduction in S and G2 phase in GSI treated cells versus controls (Figure 3C).

GSI treatment interferes with lobular breast cancer progression in vivo

Because we observed a high frequency of NOTCH1 activation in human breast cancer specimens and a proliferative block in breast cancer cell lines in vitro using NOTCH inhibitors we asked if GSI treatment would affect breast cancer progression in vivo. We used a well-established mouse model with combined conditional deletion of *Cdh1* and *Trp53* in mammary epithelial cells that develop ILC at high incidence. KEP1.11 used here is a cell line derived from these mice which grows in vitro and causes metastatic ILC with high incidence and short latency when transplanted orthotopically [32].
To address if KEP1.11 metastatic ILC is sensitive to NOTCH inhibition *in vivo*, we transplanted 10^4 cells into cleared fat pads of immune-compromised Nude mice. When tumours reached a volume of 50 mm^3, we treated the mice for 10 consecutive days with vehicle or with a 10 umole/kg of GSI. We measured tumour volumes after 10 days and observed a significant decrease in volumes in animals treated with GSI versus animals that received vehicle only (Figure 4A). The experiment was repeated for the second time where the trend of tumour volume decrease in GSI treated groups was present although not significant (data not shown). A third experiment is still ongoing to conclude this part.
We isolated tumor tissues from these mice and performed N1ICD and HES1 immunohistochemical staining on paraffin embedded tissues and observed a decrease in NOTCH1 Val1744 staining and a reduction in HES1 staining in GSI treated tumors compared to isogenic control tumors (Figure 4B and C). We also observed a significant reduction in proliferation by Ki67 tumor stainings in GSI treated mice (Figure 4B and C).

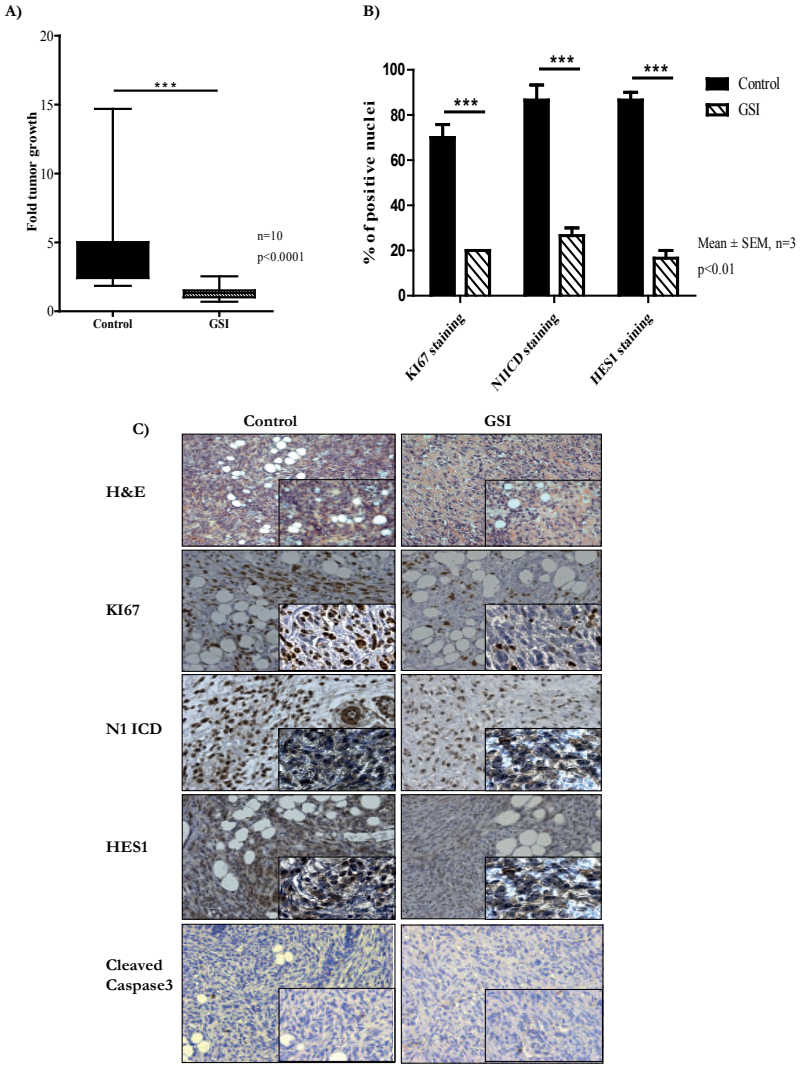

Figure 4. *Effect of GSI treatment . A) 104 KEP1.11 (metastatic mouse ILC cell lines) were transplanted into cleared fat pads of immune-compromised Nude mice. When tumors reached a volume of 50 mm3, the mice were treated for 10 consecutive days with vehicle or with a 10 umole/kg of GSI. Tumor volumes were measured by caliper after 10 days and a significant decrease was observed in volumes in animals treated with GSI versus animals that received vehicle only B) Representative immunohistochemistry images of H&E, KI67, N1ICD and HES1 staining's on isolated tumor tissues from control or GSI treated mice. We observed a decrease in KI67 (proliferation marker), N1ICD staining and a reduction in HES1 staining in GSI treated tumors compared to isogenic control tumors. C) Quantitative IHC analysis of NICD, HES and Ki-67 staining in sections from control and GSI treated mice. % of positive nuclei was calculated of the each staining. We also observed a significant reduction in all stainings in GSI treated mice. D) GSI treatment does not induce apoptosis in vivo.*

Respresentative images of cleaved Caspase3 staining of tumor tissue extracted from control and GSI treated mice.

To investigate if GSI treatment induced apoptosis in vivo, we performed immunohistochemistry against cleaved Caspase3, however very small percentage of (<1%) tumor cells were apoptotic (Figure 4C).
NOTCH inhibition is known to cause intestinal toxicity due to proliferative arrest of the intestinal crypt epithelium leading to precocious goblet cell differentiation and stem cell depletion [34]. We observed a significant increase in intestinal goblet cell differentiation but no clinical signs of gut toxicity, as observed with intraperitoneal injections of GSI (Figure 5A and B).

Figure5. *Effect of NOTCH Inhibition in the gut. A) NOTCH inhibition is known to cause intestinal toxicity due to proliferative arrest of the intestinal crypt epithelium precocious goblet cell differentiation and stem cell depletion. We observed a significant increase in intestinal goblet cell differentiation which is represented here by PAS staining of the guts from control and GSI treated mice. B) Goblets cells numbers of 10 crypts were counted from 3 different areas of each case (control and GSI treated group) and a statistical analysis was performed. We observed a significantly higher number of goblet cells (p<0.05) in guts of GSI treated mice.*

Discussion

NOTCH signaling is frequently deregulated in human cancers including breast cancer and many preclinical models have demonstrated that NOTCH inhibition may attenuate tumor growth *in vitro* and *in vivo*. The majority of these breast cancer models however are of ductal origin. Very little is known of the molecular pathways that govern lobular cancer etiology which account for about 10-15% of breast cancer cases. Here we show that the NOTCH1 receptor and a common downstream target HES1 are frequently activated in human ILC. We demonstrated that human and mouse ILC are sensitive to NOTCH inhibition *in vitro* and *in vivo* in a murine model for ILC. Altogether our findings show that NOTCH1 signaling is likely to be important for ILC etiology and that NOTCH inhibition using GSI may provide additional treatment options in the management of ILC.

To date, a number of studies have reported on the expression of NOTCH receptors and ligands in breast cancer [25, 35, 36]. Most studies have focussed on IDC. We find significantly more frequent N1ICD and HES1 expression in a high percentage of ILC compared to IDC (Table 1). There is a high concordance between cleaved NOTCH1 and expression of HES1 indicating that the NOTCH pathway is also active.

There are multiple processes controlling NOTCH activation, such as, proteolysis, glycosylation, ubiquitylation and phosphorylation. Overexpression of ligands or loss of negative regulators could lead to hyper activation of NOTCH as well. For example, it is possible that mutations in a negative regulator of NOTCH such as FBW7 and/or NUMB can lead NOTCH hyper activation. FBW7 is an E3 ubiquitin ligase, targeting NOTCH for proteasomal degradation [37]. We did not observe mutations in FBW7 in the breast cancer cell lines and patient material analysed here but whole genome sequencing efforts have reported a low frequency of FBW7 mutations in breast cancer [38]. Another negative regulator of NOTCH pathway, NUMB, is involved in targeting NOTCH for endosomal degradation [39]. Frequent loss of NUMB could therefore contribute to hyper activation of NOTCH which has been observed in breast cancer. Low NUMB expression was related with more aggressive phenotype and was a predictor of poor prognosis [40, 41].

Uncontrolled ligand driven NOTCH activity could also explain hyper activation of the NOTCH1 pathway in ILC. We have not focused on NOTCH ligands in this study. However it has previously been demonstrated by several studies that human breast cancer cases tend to have high expression levels of NOTCH ligands correlates with high receptor expression [25, 42]. Deregulated WNT signaling is a common event in mammary carcinogenesis. Many studies have shown that NOTCH ligands can be direct targets of the WNT/B-CATENIN/TCF cascade [43-46]. For example in human primary mammary epithelial cells, ectopic expression of WNT1 induces the expression of NOTCH ligands leading to NOTCH hyperactivity and mammary epithelial transformation [47]. It remains to be determined which of the above mentioned reasons lie behind our observation of high NOTCH activation in ILC.

According to the current literature NOTCH signaling plays an important role in luminal cell differentiation [30]. ILCs are usually luminal type breast cancers; a subtype mainly including hormone receptor expressing breast cancers with similar expression patterns of cytokeratins, ER and genes involved in ER activation [48] [49]. Our observation together with the published literature suggests therefore an important role of deregulation of NOTCH signaling in ILC progression due to the induced defects in luminal cell differentiation. In our data set, N1ICD correlated significantly with ER status (data not shown). Interestingly, this correlation was reported before and it was found that ER activity upregulated NOTCH1 activity [50]. Rizzo et al. exploited this by demonstrating that a therapy based on combinations of anti-estrogens (i.e tamoxifen) and a NOTCH inhibitor might be effective in the treatment of ER + breast cancers which could be an option for low or intermediate grade ILC cases. Following a similar paradigm ErbB-2 inhibition via TrastuzuMab might sensitize breast cancers to NOTCH

inhibitors. In our ILC mouse model, tumors mimic mainly high grade human ILC and are mostly ER low/negative [33]. Therefore in ILC, NOTCH inhibition may provide a good (short-term) response regardless of ER status. NOTCH signaling is known to be involved in the regulation of cell proliferation or apoptosis, depending on the cell type and context [51]. Our *in vitro* data suggests that even short term inhibition of NOTCH signaling by GSI in mouse ILC cell lines and human breast cancer cell lines results in a decrease in cell proliferation by inducing cell cycle arrest. The role of NOTCH signaling in cell cycle regulation has already been reported in different situations including breast cancer [52]. In the case of haematological malignancies, GSI treatment has been shown to induce G0/G1 arrest in a panel of T-ALL cell lines *in vitro* and *in vivo* instead of inducing apoptosis [53-55].

For our *in vivo* experiments, we made used of mouse breast cancer cell lines which were established from a mouse breast cancer model which showed strong resemblance to human ILC [32]. The tumors developed upon orthotropic transplantation of these cells into nude mice showed volume reduction upon GSI treatment. This observation correlates with our *in vitro* results, demonstrating the importance of Notch activation in the progression of this breast cancer subtype and GSI treatment as a potential therapy for ILC patients. There are many reports describing the effect of GSI inhibition in murine breast cancer models mostly resembling IDC [56]. For example in ERBB2-transgenic mouse model of breast cancer GSI decreased cell proliferation and inhibited tumor growth resulting in increased survival [57]. Watters et al., employed transcriptomics from a mouse model of breast cancer to extract signatures that predict GSI sensitivity in vivo [58]. Interestingly, Lee at al (2008) demonstrated that NOTCH upregulation caused enhanced cell proliferation *in vitro* in ER negative human breast cancer cell lines and GSI inhibition induced apoptosis in these cells.

This result is in contrast to our observations where we did rather observe proliferative arrest and not necessarily an increased apoptosis. This difference might be related to the use of different cell lines. The same group also uses an *in vivo* model, where they inoculated immunocompromised mice with tumors of an ER negative human breast cancer cell line MDA-MB-231. Intraperitoneal administration of GSI to these mice resulted in inhibition of tumour growth in a short time like observed in our study. It well established that NOTCH signaling plays an important role in cell differentiation. In the intestine GSI therapy causes stem cell depletion and secretory differentiation leading to severe dose-limiting toxicity [34]. During our experiments we did not observe any clinical signs of intestinal toxicity. We believe that this maybe due to the slow local release administration of GSI via osmotic pumps. The levels of drug in the gut might have reached to the threshold to induce goblet cell differentiation but was not enough to induce toxicity. Thus we speculate that with localized GSI treatment high enough therapeutic concentrations may be achieved in tumors without reaching the threshold for mechanism based toxicity in other organs.

GSIs are currently being evaluated in different clinical trials which include breast cancer and they appear to be successful and potent drugs that block NOTCH signalling. The

anti-tumor effects of GSI treatment on a novel in vivo ILC model that we show here warrants a further investigation into its potential for the treatment of ILC patients.

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Chapter 5

HIF-1α and NOTCH Signaling in Ductal and Lobular Carcinomas of the Breast

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Abstract

NOTCH signaling is involved in every step of metazoan development and maintenance of adult tissue homeostasis. It is frequently deregulated by mutations and overexpression in different cancer types including solid tumors such as breast cancer. Another common feature of solid tumors is hypoxia, which occurs due to defective or insufficient vascularization. Hypoxia-inducible factors (HIFs) are key regulators of the homeostatic response to low oxygen levels. HIF-1α is overexpressed in many solid tumors, including breast cancer. Hypoxia-induced stabilization of HIF transcription factors has been shown to lead to NOTCH activation in vitro in different contexts and tissues, causing differentiation arrest and induction of proliferation and migration. Since the link between HIF-1α and NOTCH signaling has hardly been studied, we set out to closely investigate associations between HIF-1α and NOTCH signaling in primary and metastatic human breast cancer specimens. Our results show that co-expression of NOTCH1 intracellular domain (N1ICD) and HIF-1α is associated with a high grade and a high proliferation rate in invasive breast cancer. HIF-1α expression was low in classic, but high in pleomorphic lobular cancers, which also frequently showed stromal HIF-1α expression. NOTCH pathway activation was associated with a poor prognosis, but NOTCH and HIF signaling did not seem to be functionally associated in breast cancer.

Introduction

NOTCH signaling serves as a short-range cell-cell communication pathway, which is highly conserved from flies to mammals. It is involved in every step of metazoan development and maintenance of adult tissue homeostasis. NOTCH proteins are single pass type transmembrane receptors consisting of extracellular ligand binding and cytoplasmic signal transduction domains [1]. Canonical NOTCH signaling is regulated by proteolysis and initiated by the interaction of transmembrane bound ligands and receptors on neighboring cells. Upon the final intramembranous cleavage of receptors, the NOTCH intracellular domain (NICD) is released and translocated to the nucleus. In the nucleus, NICD interacts with the transcription factor CSL (CBF1 in humans; RBP-J_k in mice) to activate the HES/HEY family of genes, which are involved in growth, proliferation, differentiation, and survival [2]. NOTCH signaling is frequently deregulated by mutation and overexpression in different cancer types [3]. Therefore, targeting NOTCH in human malignancies could be a powerful approach to counteract tumor progression.

The NOTCH pathway has also been shown to play an important role in breast cancer. NOTCH1 was the first member of the family identified in breast cancer and the NOTCH1 locus was also identified as a Mouse Mammary Tumor Virus (MMTV) insertion site in MMTV-Neu tumors [4]. Based on previous research, we have proposed that dose and context dependency of NOTCH pathway activation may lead to different phenotypes in different tissues [5]. Immunohistochemistry performed on human breast cancer specimens revealed frequent overexpression of NOTCH1 [6], NOTCH3 [7-9] and the NOTCH ligand JAGGED1 in most cases, together with loss of expression of NUMB, a negative regulator of NOTCH [10]. NOTCH family members seem to have different roles in cancer. For example, high NOTCH2 expression in breast cancer has been shown to be correlate to a higher chance of survival [11]. In vivo studies performed in mice supported the tumor promoting role of NOTCH1, NOTCH3 and NOTCH4 [7, 12, 13].

Low oxygen tension (hypoxia) is a common feature of solid tumors and it can occur due to defective or insufficient vascularization. Hypoxia-inducible factors (HIFs) are key regulators of the homeostatic response to low oxygen levels. Hypoxia and HIF-1α, the hypoxia response-regulating unit of the HIF complex, have been proposed to play a role in breast carcinogenesis [14] and to affect breast cancer prognosis [15, 16]. Hypoxia-dependent NOTCH activation has been observed in different contexts and tissues [17-19]. Recently, Chen et al. demonstrated that hypoxia-induced stabilization of HIF transcription factors leads to expression of the NOTCH target genes HES1 and HEY1 in human breast cancer cell lines [20]. In another study, Xing et al. reported increased JAGGED2 (a NOTCH ligand) and NOTCH1 intracellular domain (N1ICD) expression in the hypoxic invasive front of the tissues (breast cancer specimens) investigated [21]. Given these interesting results, which indicate a potential link between these two oncogenic pathways in breast cancer, we investigated the associations between HIIF-1α

and N1ICD expression in primary and metastatic human breast cancer specimens, in conjunction with downstream targets of both pathways.

Materials and Methods

Tissue samples

Formaldehyde-fixed paraffin embedded breast cancer tissue blocks of 449 cases were collected from the archives of Departments of Pathology of the University Medical Center Utrecht (Utrecht, the Netherlands) and the Radboud University Nijmegen Medical Centre (Nijmegen, the Netherlands) to prepare tissue microarrays (TMAs). This series was enriched in lobular carcinomas because of a special interest in this type of cancer by our research group. Typing was done according to the WHO, and grading was done according to the Nottingham scheme. The mitotic activity index (MAI) was assessed as previously described [22]. Representative areas containing morphologically well-defined tumor tissues were identified in haematoxylin-eosin stained reference slides by a pathologist (PJvD). Tissue cylinders of 0.6 mm were transferred from these tumor areas in each donor block to the recipient block using a tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA). Four μm thick serial sections were cut from the recipient blocks and transferred to Superfrost + slides to produce TMAs (Menzel and Glaeser, Germany) for immunohistochemistry. The use of anonymous or coded leftover material for scientific purposes is part of the standard treatment contract with patients in The Netherlands [23]. Ethical approval was therefore not required. Overall survival data were obtained from the Comprehensive Cancer Centre of The Netherlands (Integraal Kankercentrum Nederland, IKNL).

Material of distant metastases (including brain, lung, skin, liver and GI tract) was available for 44 out of 449 cases primary breast cancer cases (19 lobular, 6 ducto-lobular and 19 ductal). These were used to investigate the difference in HIF-1α and N1ICD expression in primary tumors and its corresponding metastases.

Immunohistochemistry

Immunohistochemistry was carried out for the following proteins: N1ICD, HES1, HIF-1α, and the HIF downstream proteins CAIX and GLUT1. After deparaffination and rehydration, the TMA slides were immersed in a buffer solution containing 0.3% hydrogen peroxidase for 15 minutes to block endogenous peroxidase activity. Antigen retrieval was obtained by boiling for 20 min in 10mM citrate buffer pH 6.0 for GLUT1, CAIX and N1ICD staining, or Tris/EDTA buffer pH 9.0 for HES1 and HIF-1α staining. A cooling off period of 30 min was applied before pre-incubation with 1:50 normal goat serum in PBS (pH: 7.4), containing 0.1% sodium azide and 1% BSA to block unspecific binding sites. This was followed by primary antibody incubation: polyclonal rabbit activated NOTCH1 antibody (Abcam, Cambridge, UK) in 1:500, HES1 antibody (Millipore, Billerica, MA, USA) 1:600, GLUT1 (DAKO, Glostrup, Denmark) in 1:200; CAIX (Abcam) 1:1,000 in PBS/1%BSA, HIF-1α (BD Transduction Labs, Breda, The Netherlands). The slides were incubated with antibody solutions either for 1 hour at

room temperature (GLUT1, CAIX) or overnight at 4˚C (HIF-1α, HES and NOTCH1). After that, the sections were incubated with Brightvision poly-HRP anti-mouse, rabbit, rat (DPVO-HRP, Immunologic, Duiven, The Netherlands) or the Novolink kit (Leica, Rijswijk, The Netherlands) (in the case of HIF-1α) and developed with diaminobenzidin, counterstained with hematoxylin, dehydrated in alcohol, and sealed with a coverslip. Throughout the immunohistochemical analyses, negative controls were obtained by omitting the primary antibodies. For NOTCH1 and HES1 staining, normal breast tissue was used as a positive control. For GLUT1 staining, positive erythrocyte staining was used as an internal control. For CAIX and HIF-1α, a breast cancer case was included that was previously proven to be positive for these markers.

Scoring

Scoring of immunohistochemistry was done in a blinded fashion with respect to patient characteristics and other staining results. N1ICD and HES1 staining in more than 10% of nuclei was considered as positive. The percentage of nuclei positive for HIF-1α was estimated as well. The staining was considered positive when ≥1% nuclear staining was observed, as described before [24]. GLUT1 and CAIX expression were scored positive when membrane staining was observed. HIF-1α expression in the stroma was also scored positive when frequent nuclear staining in fibroblasts was observed.

Statistics

The SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Pearson's Chi-square test was used to examine associations between categorical variables. Percentages of nuclei expressing HIF-1α and N1ICD in primary tumors and their corresponding metastasis were compared by a paired Student's t-test. The graphs were made using GraphPad Prism 5 (GraphPad Software Inc., CA, USA). Survival analysis was performed by plotting Kaplan-Meijer curves and Log rank test. Two-sided p-values below 0.05 were considered as statistically significant. Multivariate survival analysis was performed using Cox regression, using entry and removal limits of 0.05 and 0.10, respectively.

Results

Associations between HIF-1a and NOTCH pathways, and clinicopathological features

Table 1 summarizes the clinicopathological characteristics of the patient material used in this study. Table 2 shows the association between HIF-1α and N1ICD and Table 3 summarizes the associations between the expressions of the proteins studied. The expression of HIF-1α and that of its downstream targets CAIX and GLUTI ($p<0.001$ for both, Table 3) were significantly associated, as expected, as were those of N1ICD and its downstream target HES1 ($p=0.004$). HIF-1α expression correlated with neither N1ICD ($p=0.435$, Table 2) nor HES1 expression, and N1ICD expression did not correlate with the expression of HIF-1α targets. The only significant correlation observed was between GLUT1 and HES1 expression ($p = 0.003$) (Table 3 & 4). Also,

no significant associations were noted between the expression of proteins in these pathways, when investigated in the N1ICD negative subgroup (data not shown).

Feature	Grouping	N or value
Age (years)	**Mean**	60
	Range	28-88
Histological Type	IDC	290
	ILC	119
	other	40
Tumour size (cm)	≤2	179
	>2 and ≤5	203
	>5	46
	missing	21
Mitotic Index (per 2mm²)	≤ 12	204
	≥ 13	230
	missing	15
Lymph node status	Negative	139
	Positive	193
	missing	117

Table1. *Clinopathological characteristics of 449 invasive breast cancer patients included in this study*

Co-expression of N1ICD and HIF-1α was significantly associated with a high tumor grade ($p<0.001$) and a high mitotic activity index (MAI; $p<0.001$) (Table 5). No other significant correlations with clinicopathological features were observed.

	HIF-1α expression		
N1 ICD expression	**-**	**+**	p-value
-	50	31	0.435
+	222	128	

Table 2. *Association between expression of HIF-1a and N1ICD (chi-square test)*

Associations between HIF-1a and NOTCH pathway proteins in primary breast cancers and distant metastases

There were no significant associations between the expression of N1ICD and HIF-1α pathway proteins in distant metastases (data not shown). However, primary breast tumors expressed on average higher levels of N1ICD than their corresponding metastases ($p<0.05$), especially in the cases that metastasized to brain ($p=0.001$) and skin ($p<0.05$) (Figure 1).

	CAIX	GLUTI	HES1
HIF1-α	p < 0.001	p < 0.001	p= 0.313
N1ICD	p = 0.283	p = 0.279	p= 0.004

Table 3. *Summary of associations between expression of HIF-1a and N1ICD on the one hand and their downstream targets CAIX, GLUT1 and HES1 on the other hand in invasive breast cancer (chi-square test).*

Table 4. *Association between expression of GLUT1 and HES1 (chi-square test)*

	GLUT1 expression		
HES1 expression	-	+	p-value
-	184	67	0.003
+	167	31	

HIF-1a expression in lobular breast carcinomas and surrounding stroma cells

High HIF-1α expression in tumor cells was observed more often in ductal carcinomas (109/277, 39%) and the pleomorphic variant of lobular breast cancer (21/54, 38.8%) as compared to classic lobular cancer cases (1/34, 2.9%) (p<0.001). Strikingly, pleomorphic lobular cases more frequently exhibited stromal HIF-1α expression as compared to classic lobular breast carcinomas (42.5 % vs 0%, p=0.001) (Table 6). Figure 2 shows representative images of HIF-1α expression in tumor cell nuclei (A) and in stromal fibroblast nuclei (B). Stromal HIF-1α correlated to HIF-1α expression in the tumor (p<0.001).

Associations between HIF-1a and NOTCH pathway proteins and survival

In all patients, there was no difference in survival between patients with no N1ICD and HIF-1α expression and those with either N1ICD expression alone or N1ICD/HIF-1α co-expression (p=0.708). The same applied to the subgroups of classic and pleomorphic ILC (Figure 3). Stromal HIF-1α expression in both classic and pleomorphic lobular breast cancer patients also had no prognostic value. In the entire group, patients with HES1 expression exhibited a significantly worse survival as compared to the low HES1 expressing group (p=0.03) (Figure 4).

Upon Cox regression analysis, lymph node status and mitotic index were found to act as independent prognosticators, and none of the other variables had additional prognostic value. HES1 was, however, close to having additional prognostic value (p=0.089).

Discussion

The NOTCH and HIF signaling pathways are highly conserved through evolution, and play a role in various cellular processes [25, 26]. Several studies have demonstrated the overlapping effects of hypoxia-induced expression of HIFs and NOTCH signaling in normal development and various cancers [20, 27, 28] . It has been suggested that NOTCH signaling is aberrantly activated during tumor progression, and that hypoxia might further stimulate its activation [20]. For example, NOTCH1 mRNA levels

were found to increase upon stabilization of HIF-1α in melanoma cell lines [19], and NOTCH1 signaling was found to be upregulated in lung cancer cell lines cultured in hypoxic conditions [17]. Very recently, Xing et al. (2011) investigated a large group of breast cancer patients and observed a strong upregulation of JAGGED2, a NOTCH ligand, and NOTCH signaling at the hypoxic invasive tumor front, although they did not show a firm correlation between HIF and NOTCH signaling by immunohistochemistry analysis in patient material [21]. Previously, Chen et al. (2010) demonstrated by chromatin immunoprecipitation (ChIP) that hypoxia-induced HIF-1α binds to the HES1 promoter, thereby inducing its activity [20]. These researchers claimed that the mechanism behind this observation likely involves an interaction of HIFs with the NOTCH co-activator MAML1, which potentiates NOTCH activation.

Feature	**Coexpression of HIF-1α and N1ICD**			
	N1ICD↓ HIF-1α↓	**N1ICD↓ HIF-1α↑**	**N1ICD↑ HIF-1α↓**	**N1ICD ↑ HIF-1α↑**
Histological type				
Ductal	40	31	135	84
Lobular	26	6	70	30
Other	1	3	16	11
Histologic grade				
1	13	5	46	9
2	21	7	88	41
3	30	27	78	**72***
Tumour size (cm)				
≤2	30	15	94	50
>2 and ≤5	32	22	93	59
>5	4	2	26	14
Mitotic Index (per 2mm²)				
≤ 12	29	11	128	46
≥ 13	36	28	87	**76***
Lymph node status				
Negative	34	21	106	55
Positive	27	19	97	64

Table 5. *Associations between HIF-1a and NOTCH1 intracellular domain (N1ICD) co-expression and clinicopathologic features (* means p<0.001)*

Our immunohistochemistry results did not show significant correlations between HIF-1α and N1ICD expression, indicating no direct functional effect of HIF-1α on the activation of NOTCH in invasive breast cancer. There was also no association observed between HIF-1α and HES1, suggesting that HIF-1α might not directly induce HES1 expression, as was suggested by the ChIP experiments of Chen et al. [20]. We have no mechanistic explanation for the association between GLUT1 and HES1 expression, an

observation that may be haphazard.

	HIF-1α expression in tumor cells			HIF-1α expression in fibroblasts		
	-	+	p-value	-	+	p-value
Pleomorphic ILC	33	21	0.001	31	23	0.001
Classic ILC	33	1		34	0	

Table 6. *HIF-1a expression in tumor nuclei and stromal fibroblasts of pleomorphic versus classic invasive lobular cancers*

Interestingly, co-expression of HIF-1α and N1ICD was significantly associated with high tumor grade and high mitotic activity index, suggesting that activation of both signaling pathways might be leading to a more aggressive phenotype. However, this hypothesis should be investigated and supported further by functional evidence, especially since HIF-1α and N1ICD co-expression had no prognostic value, and metastases expressed lower levels of N1ICD as compared to the primary tumors. Our group has previously shown that the immunophenotype of distant breast cancer metastases can be different from that of the primary tumor [29] with prognostic impact [29, 30]. NOTCH targeting may therefore be less effective in brain and skin metastases in breast cancer patients, and may be especially effective in preventing local recurrences and metastases to sites other than brain and skin. Previously, a similar observation was reported for NOTCH expression in human colorectal cancer [31].

It was interesting to note the prognostic value of expression of HES1 expression, a downstream target of all NOTCH members [32]. N1ICD itself had no prognostic value, which may be explained by the fact that it is highly expressed in the normal breast, where it might not be functional. The association of high HES1 expression with a poor prognosis, which had an almost additional prognostic value to lymph node status and mitotic index, may indicate that NOTCH1 downstream activation does lead to more aggressive behavior of breast cancer. However, we cannot exclude that NOTCH family members other than NOTCH1 may have caused the activation.

It is well established now that the tumor stroma is important for regulating tumor growth [33]. The role of fibroblasts, the principal cellular components of connective tissues, in cancer progression is increasingly recognized. Recently, Chiavarina et al. (2010) speculated, based on *in vitro* and *in vivo* experiments, that HIF-1α might have a tumor promoting role in breast cancer associated fibroblasts [34]. Our observation of strong stromal HIF-1α expression, particularly in pleomorphic lobular cases, is interesting in this respect. Novel therapies targeting HIF-1α may add to the current treatment strategies of pleomorphic cases [35].

In conclusion, co-expression of N1ICD and HIF-1α is associated with a high grade and a high proliferation rate in invasive breast cancer, and activation of the NOTCH pathway is associated with a poor prognosis. HIF-1α expression is low in classic and high

in pleomorphic lobular cancers, the latter of which also frequently show stromal HIF-1α expression. However, N1ICD and HIF-1α do not seem to be functionally related in breast cancer. Primary breast cancers express higher levels of N1ICD than their corresponding metastases, especially those in the brain and skin, implying that NOTCH targeting may especially be effective in preventing local recurrences and metastases at sites other than brain and skin.

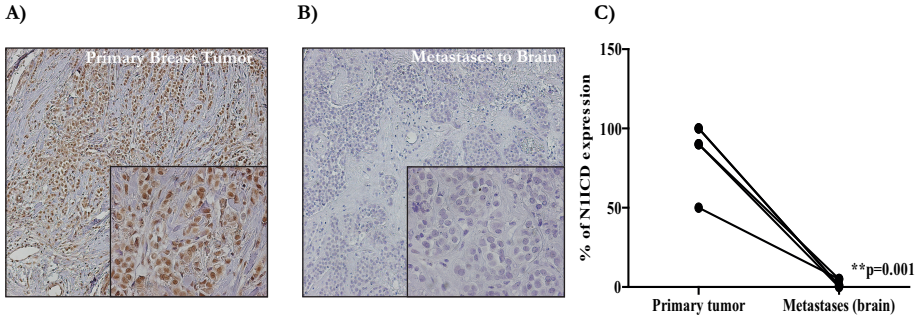

Figure 1. *High N1ICD expression in primary tumors which metastasized to brain*
A representative image of N1ICD staining in a primary breast tumor case (10X and 40X) A) and its corresponding metastases B) to the brain. C) N1ICD expression in paired primary breast tumors and corresponding brain metastases (p<0.001).

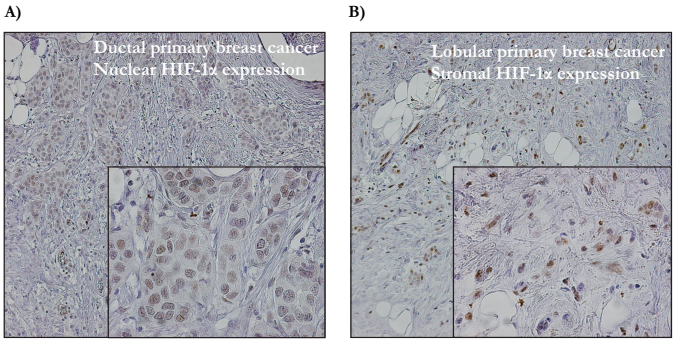

Figure 2. *HIF-1a expression in ductal and lobular breast carcinomas and surrounding stroma. Representative images of HIF1-a staining (10X and 40X) in A) ductal breast carcinoma and B) lobular breast carcinoma.*

Acknowledgements

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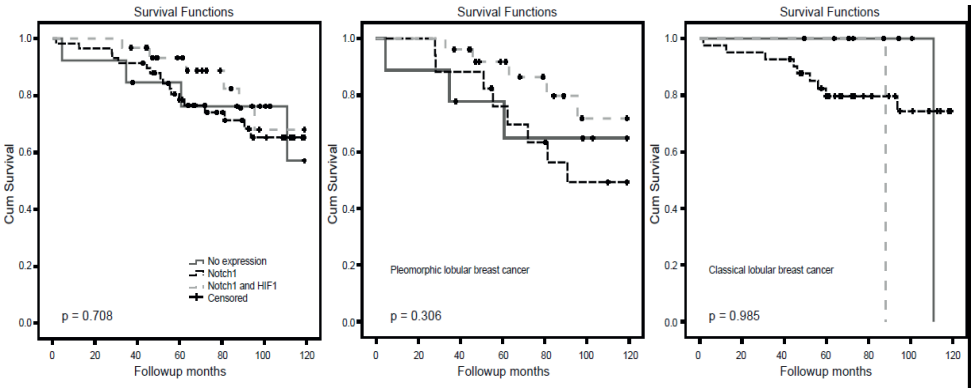

Figure 3. *Survival curves of breast cancer patients according to HIF-1a/N1ICD status (left all patients, middle and right subgroups of pleomorphic and classic ILC, respectively). There were no significance differences in survival.*

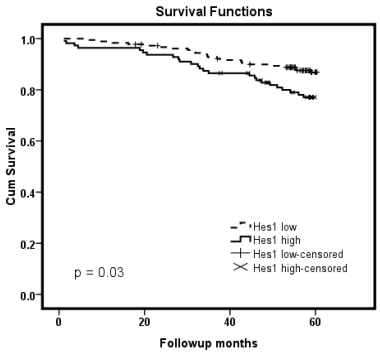

Figure 4. *Survival curves of breast cancer patients according to HES1 status. High HES1 expression, indicating activation of the NOTCH pathway, is associated with worse survival.*

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Chapter 6

p53 Mutations in Classic and Pleomorphic Invasive Lobular Carcinoma of the Breast

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Abstract

p53 is a tumor suppressor gene that is commonly mutated in human cancers. Although alterations in p53 are common in breast cancer, few studies have specifically investigated p53 mutations in the breast cancer subtype invasive lobular carcinoma (ILC). Recently reported conditional mouse models have indicated that functional p53 inactivation may play a role in carcinogenesis of ILC. Since reports on the detection of p53 mutations in the relatively favorable classic and more aggressive pleomorphic variants of ILC (PILC) are rare and ambiguous, we performed an extensive analysis to determine p53 status in these breast cancer subtypes. To increase our understanding of p53-mediated pathways and the roles the may play in the etiology of classic ILC and PILC, we investigated p53 mutations and p53 accumulation in a cohort of 22 cases of classic and 19 cases of PILC by direct DNA sequencing and immunohistochemistry. We observed 11 potentially pathogenic *TP53* mutations, of which 3 were detected in classic ILC (13.6%) and 8 in PILC (42.1%; p=0.04). Mutations that affected structure and protein function were significantly associated with p53 accumulation. However, p53 accumulation was not significantly different between classic and pleomorphic ILC cases. In conclusion, p53 mutations seem to occur more frequently in PILC than classic ILC.

Introduction

The tumor suppressor p53 was first described in 1979 as a key cell cycle regulator. Upon cellular stress, the p53 signaling pathway turns on the expression of genes including inhibitors of cell cycle, DNA repair, and apoptosis [1, 2]. Inactivating alterations in the p53 gene are commonly observed in human cancers, resulting in suppression of the regulatory functions of p53, which contributes to transformation of cells. Mutations in p53 are observed in breast cancer, however with a lower frequency (~ 20%) compared to other solid tumors [2]. Although it has been well established that p53 mutations correlate with high grade and triple negativity [3, 4], the p53 mutation spectrum across the various different histological types of breast cancer has not been well defined.
Invasive lobular cancer (ILC) accounts for approximately 10% of breast cancers [5]. Based on their molecular profile, most ILC belong to the luminal–type breast cancers. Within ILC, several subtypes can be discerned: 1) the classic type composed of small regular cells with frequently intracytoplasmic vacuoles, small nuclei and a typical trabecular infiltration pattern with dissociated cells or forming single files, often in targetoid patterns around uninvolved ducts and with low mitotic rate; 2) the better demarcated alveolar type exhibiting small round aggregates of 20 or more cells with typical lobular cytology; 3) the also better demarcated solid variant consisting of more solid sheets of cells with little intervening stroma, more mitoses and often some more atypia; 4) the pleomorphic variant that exhibits the growth pattern of classical lobular carcinoma throughout but with polygonal, eccentric pleomorphic nuclei and more frequent mitoses [6]. The pleomorphic variant of ILC (PILC) accounts for less than 1% of all breast carcinomas and not more than 10% of all ILC [7]. It has a poorer prognosis compared to classic ILC (5, 8). Several studies have addressed the molecular and histological aspects of PILC as a separate entity. Immunohistochemical analyses demonstrated that PILCs frequently express estrogen (ER) and progesterone (PR) receptors and are mostly E-cadherin negative, Her2 positive and occasionally p53 positive [8-12]. Although expression of gross cystic disease fluid protein (GDCFP) has been used to facilitate differential diagnosis between classic ILC and PILC, reliability is variable [11-14]. Little is known about p53 mutation status in PILC. In view of the higher nuclear grade and poorer prognosis, one would expect a higher frequency of p53 mutation in PILC. Interestingly, conditional knock-out mouse models have indicated that functional inactivation of p53 may play a role in carcinogenesis and progression of mouse PILC [15, 16]. In the present study, we have investigated the p53 mutation status in a series of 41 ILC cases including 22 classic and 19 pleomorphic subtypes to advance current knowledge on these variants of ILC.

Materials and Methods

Patients

Archival material from 41 breast cancer patients with lobular carcinoma was collected from the Pathology departments of the University Medical Center Utrecht, Utrecht, the Netherlands and Radboud University Nijmegen Medical Centre, Nijmegen,

The Netherlands, and the Institute of Pathology, Paderborn, Germany. ILC and PILC were identified on Hematoxylin and Eosin (H&E) stained reference slides from formaldehyde-fixed paraffin embedded breast cancer tissue blocks of 41 cases by an experienced pathologist (PJvD), considering cases with nuclear atypia score 3 as PILC. Use of anonymous or coded left over material for scientific purposes is a part of the standard treatment contract with patients in our hospitals [17].

DNA extraction

After de-paraffinization of the slides by standard methods, the relevant area from each slide (as identified on corresponding H&E stained sections) was scraped off with a scalpel and suspended in lysis buffer (Tris / HCl buffer pH 8.0 with Tween). Then, proteinase K was added (concentration) and the samples were incubated for 1 hour at 56°C. After that, samples were incubated at 95°C for 10 minutes to inactivate proteinase K.

Sequencing and mutation analysis

For the detection of mutations, genomic DNA was amplified with primers flanking exons 4, 5, 6, 7, 8 and 9 of the TP53 gene (Tables 1).

Exon	Sequence
4.1 FW	5' CTG GTC CTC TGA CTG CTC 3'
4.1 RV	5' GAC AGA AGA TGA CAG GGG 3'
4.2 FW	5' AGC TCC CAG AAT GCC AGA G 3'
4.2 RV	5' TGA AGT CTC ATG GAA GCC 3'
5.1 FW	5' CCG TGT TCC AGT TGC TTT ATC 3'
5.1 RV	5' GCT GTG ACT GCT TGT AGA TG 3'
5.2 FWa	5' TCA ACA AGA TGT TTT GCC AAC TGG 3'
5.2 FWb	5' ACA AGA TGT TTT GCC AAC TG 3'
5.2 RV int	5' GAG CAA TCA GTG AGG AAT CAG 3'
6 FW	5' TCC CCA GGC CTC TGA TTC 3'
6 RV	5' CTG ACA ACC ACC CTT AAC C 3'
7 FW	5' CTT GCC ACA GGT CTC CCC AA 3'
7 RV	5' GCG GCA AGC AGA GGC TGG 3'
8 FW	5' CCT TAC TGC CTC TTG CTT C 3'
8 RV	5' TAA CTG CAC CCT TGG TCT C 3'
9 FW	5' GTT ATG CCT CAG ATT CAC T 3'
9 RV	5' TGA GTG TTA GAC TGG AAA C 3'

Table 1. *Summary of primer sequences*

The PCR conditions were set up as follows; initial denaturation at 94°C for 3 min, 35 cycles at 94°C for 1 min (denaturation), 60°C for 30 sec (annealing) and 72°C for 45 sec (extension), followed by a final extension at 72°C for 5 min. Then, PCR products were sequenced in both sense and antisense directions using the BigDye Terminator v1.1 sequencing kit on ABI 3130 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The sequences were analyzed using Mutation Surveyor

software (SoftGenetics, LLC., State College, PA, USA).

Immunohistochemistry

Four μm thick sections were cut from the paraffin blocks and transferred to Superfrost+ slides (Menzel and Glaeser, Braunschweig, Germany). Citrate buffer was used for antigen retrieval Immunohistochemistry was then performed with a mouse monoclonal p53 antibody, clone BP53-12-1, 10 mg/mL stock, (MU 195-UC, BioGenex**,** San Ramon, CA, USA; 1:100) on an automated immunostainer (Bond-MaX, Leica, Bannockburn, IL).. Slides were counterstained with hematoxylin, dehydrated, and cover-slipped. Throughout the immunohistochemical analysis, negative controls were obtained by omitting the primary antibody and staining of a colon cancer tissue with a verified p53 mutation was used as a positive control. Scoring of the stained nuclei was performed by an experienced pathologist (PJvD). p53 was regarded as accumulated when ≥5% of nuclei were stained. p53 was regarded as wild-type when approximately 1% of nuclei showed expression.

Statistical analysis

Chi-Squared test, or, when appropriate, Fischer's Exact test was applied to compare frequencies between groups with the SPSS 15.0 software package (IBM), regarding p-values <0.05 as significant.

Results

We identified the pleomorphic ILC variant using H&E staining, based on a classical trabecular ILC growth pattern, but with polygonal eccentric pleomorphic nuclei and more frequent mitoses (cases with nuclear atypia score 3). We have not observed a significant increase in HER2 expression in PILC when comparing with classical ILC in our samples (data not shown).
The rationale behind our investigation of p53 in classic ILC and PILC is gaining a new insight into distinctive gene alterations which give rise to these two different subtypes of ILC.

p53 mutations

To investigate the incidence of *TP53* mutations in classic ILC and PILC, we performed PCR on exons 4 – 9 (conserved midregion) of *TP53* for 41 ILC cases (22 classic ILC and 19 PILC). Direct DNA sequencing was subsequently performed on PCR products. Overall, we detected 11 mutations (of which 1 novel and 10 previously reported) and 2 validated polymorphisms in 41 ILC cases (Tables 2 and 3). One out of 11 mutations was located in an intron and 10 mutations were located in coding regions. Using the freely available IARC TP53 database, we have scrutinized the following; the functions of the domains in which the mutated residues are located, the known functions of the wild-type residues, the effect of the mutations, the predicted effect on splicing, functional predictions based on the structure change and previously reported tumor sites (Table 2) [18, 19].

Case	Location	Genomic Description	AA Change	Domain Function[a]	Residue Function[b]	Effect[c]	Predicted Effect on Splicing[d]	Structure-Function[e]	Observed Tumor Sites	p53 expression[f]
ILC-1	Exon5	g.12368C>A	p.S127Y	DNA Binding	Buried	Missense	No significant change	Nonfunctional	Mainly Gallbladder, incl. Breast.	100%
ILC-2	Exon6	g.12696A>T	p.R209S	DNA Binding	Exposed	Missense	New Site	Functional	Mainly Liver	1%
ILC-3	Exon6	g.12683A>G	p.Y205C	DNA Binding	Buried	Missense	New Site	Nonfunctional	Mainly in Urinary Tract, incl. Breast	67%
PILC-1	Exon5	g.12741G>A	p.E224E	DNA Binding	Exposed	Silent	Site Broken – New Site	NA	Mainly Bones	1%
PILC-2	Exon8	g.13798C>T	p.R273H	DNA Binding	DNA Binding	Missense	No significant change	Nonfunctional	Mainly Lungs, incl. Breast	20%
PILC-3	Intron7	g.13440C>T	--	NA	NA	Intronic	No significant change	NA	Mainly Nasal Cavity	0%
PILC-4	Exon5	g.12492C>T	p.H168H	DNA Binding	Partially Exposed	Silent	No significant change	NA	Mainly CervixUteri, incl. Breast	3%
PILC-5	Exon5	g.12469G>A	p.A161T	DNA Binding	Buried	Missense	No significant change	Nonfunctional	Mainly in Meninges, incl. Breast	30%
PILC-6	Exon5	g.12442C>T	p.P152S	DNA Binding	Partially Exposed	Missense	No significant change	Nonfunctional	Mainly in Renal pelvis, incl. Breast	7%
PILC-7	Exon4	g.11387A>G	p.Q52Q	NA	NA	Silent	New Site	NA	--	3%
PILC-8	Exon5	g.12543C>T	p.S185S	DNA Binding	Exposed	Silent	New Site	NA	Mainly Head & Neck, Nos	4%

Table 2. *p53 mutation analysis results of Classic and Pleomorphic Lobular Breast Cancer [18] (Version of the database; R15, November 2010)*

a.Domain Function; Function of the domain in which the mutated residue is located.

b.Residue Function; Known function of the wild-type residue (NA=Not Available)

c.Effect; Effect of the mutation. The terms occurring in this column are: missense (change of one amino-acid) and silent (no change in the protein sequence).

d.Predicted Effect on Splicing; This is the predicted effect of the mutation on splicing based on two splicing prediction tools NNSPLICE0.9 and HSF V2.3. No significant change: no change in the wild type splice motif. New site: the mutation created a splice motif not present in the wild type sequence. Site broken: the mutation removed a splice motif that was identified in the wild type sequence.

e.Structure-Function; Functional predictions derived from a computer model that takes into account the 3D structure of wild type and mutant proteins and is trained on the transactivation dataset from Kato et al. (2003). Mutations are classified as "functional" or "non-functional".

f.p53 Expression; The results of immunohistochemistry analysis of p53 protein expression for these cases is represented on this column. (Percentage of cells expressing p53)

This data summarized in Table 2 allowed us to predict the pathogenicity of the observed mutations. We conclude that all of the 11 mutations found could potentially be pathogenic based on the mentioned criteria above.

Next, we evaluated the distribution of these potentially pathogenic mutations over classic and pleomorphic ILC variants. Eight of the 19 PILC cases (42.1%) exhibited a potentially pathogenic mutation which is significantly more often when compared to the percentage of potentially pathogenic mutations found in classic ILC cases (3 mutations (missense) observed in 22 classic ILC cases (13.6 %; $p \leq 0.05$)) (Table 4). We have also observed two previously reported and validated polymorphisms among our samples. These were distributed similarly over the classic and pleomorphic ILC variants (Table 3).

#	Location	Genomic Description	Aminoacid Change	Domain Function[b]	Effect[2c]	Predicted Effect on Splicing[d]	Observed in
1	Exon4	g.11339G>A	36P>P	Transactivation	Silent	NA	3 PILC and 3 ILC cases
2	Exon4	g.11446C>G[a]	72P>R	SH3-like/Pro-rich	Missense	New Site	11 PILC and 18 ILC cases

Table 3. *p53 mutation analysis results of Classic and Pleomorphic Lobular Breast Cancer [18] (Version of the database; R15, November 2010)*
a Several studies showed its association with breast cancer in different populations.
b Domain Function; Function of the domain in which the mutated residue is located.
c Effect; Effect of the mutation. The terms occurring in this column are: missense (change of one amino-acid) and silent (no change in the protein sequence).
d Predicted Effect on Splicing; This is the predicted effect of the mutation on splicing based on two splicing prediction tools NNSPLICE0.9 and HSF V2.3. No significant change: no change in the wild type splice motif. New site: the mutation created a splice motif not present in the wt sequence. Site broken: the mutation removed a splice motif that was identified in the wt sequence. (NA=Not available)

p53 expression and accumulation

p53 immunostaining was performed to investigate the correlation of mutational status and effects on protein expression. Immunohistochemistry scores and mutation data of each case are summarized in Table 2. We observed a variation in the immunohistochemistry scores of p53 in both pleomorphic and classic cases. In normal breast tissue, p53 staining is observed in a small percentage (approximately 1%) of the cells. Therefore, 5% positivity was chosen as a value to distinguish normal, wild-type p53 expression and mutated p53 overexpression. Overall, 9/41 cases (21.9%) showed p53 accumulation, while 6 cases showed absence of expression. Of these 15 cases, 6 were ILC and 9 PILC ($p=0.157$). In conclusion, p53 accumulation was not associated exclusively with a specific ILC variant.

	Potentially Pathogenic p53 Mutations		
	+	-	
Classic ILC	3 (13.6 %)	19 (86.4 %)	Total 22
PILC	8 (42.1 %)	11 (57.9 %)	Total 19

Table 4. *Correlation of potentially pathogenic p53 mutations and Classic and Pleomorphic Invasive Lobular Carcinomas ($p < 0.05$ *)*

Correlation of p53 accumulation and mutation

Because a large body of literature has shown that p53 accumulation can be caused by mutations in *TP53*, we set out to investigate the correlation between p53 accumulation and mutation. In total, 5 out of 9 cases that showed p53 accumulation based on immunohistochemistry harbored mutations (Table 5).

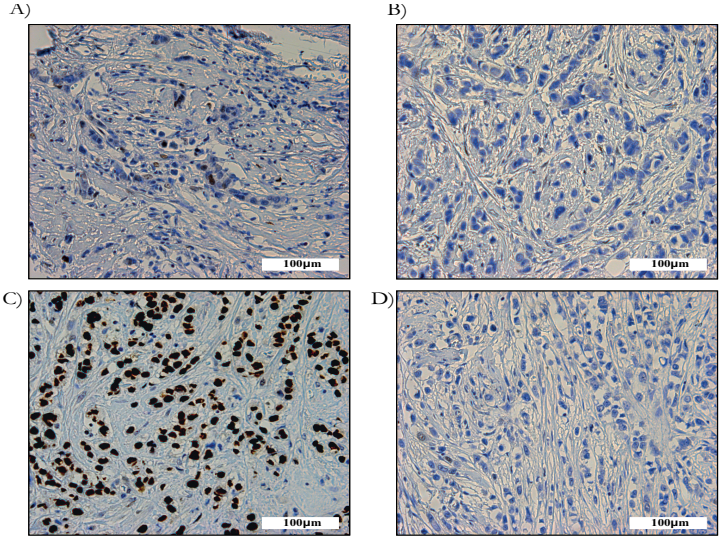

Figure 1. *p53 expression in PILC. A-D. Representative examples of p53 expression of PILC cases (a) wild-type; 1% positive nuclei (b) no expression and absence of mutations (c) 'g.12368C>A' mutation resulting in 100% p53 overexpression (d) 'g.13440C>T' mutation occurring in an intron site and resulting in absence of p53 expression*

Interestingly, we detected potential pathogenic mutations in 4 of the 32 cases without p53 accumulation (wild-type staining + 0% staining) (p=0.01), while 1 of 6 cases showed a pathogenic mutation in the absence of p53 staining (0% staining). However, the 5 remaining cases with 0% staining had one of the two validated polymorphisms (Number 2 in Table 3). In summary, we observed a significant correlation of p53 accumulation with potentially pathogenic mutations (Table 5).

		Mutations causing a predicted defect in p53 structure	
		-	+
p53 Accumulation	-	32	0
	+	4	5

Table 5. *Correlation between p53 mutations that give rise to a non-functional structure and p53 accumulation*

Discussion

Pleomorphic ILC was first described in 1982 as a variant of infiltrating lobular carcinoma [20]. Even though the morphology of PILC with high nuclear atypia is distinctive, its feature of high frequency of multicentrity and bilaterality seem to be similar to the classic ILC [21]. The report of Weidner and Semple (1992) is one of several reports demonstrating the aggressive clinical behavior and poorer prognosis of patients with PILC in comparison to patients with ILC [9]. Therefore, despite the similarities between them, the question mark on the genetic pathways through which each variant evolves still remains. The *TP53* tumor suppressor gene has been an interesting target to investigate in invasive breast cancer since it is very frequently altered in other human cancers [22]. Many research groups have investigated the distribution of p53 mutations and its correlation with immunohistochemistry in invasive carcinomas [23-27] but data focusing on different variants of ILC are limited. Therefore our aim was to study the mutational status of p53 in classic and pleomorphic ILC to gain a better understanding of the molecular changes occurring in this gene, which possibly contribute to the development of these subtypes and the potential of it as a tool to differentially diagnose ILC *versus* PILC.

In the present work, we studied 41 ILC cases for p53 mutations and accumulation in relation to the classic and pleomorphic variants. Eleven mutations were detected in 41 cases studied (26%) which is in line with the literature which states that the overall frequency of p53 mutations in breast cancer is approximately 20% [28]. Almost all the observed mutations locate in the highly conserved DNA-binding domain of the protein [18] (Table 2). Interestingly, our mutation analyses reveal that PILC is associated with a higher frequency (42.1 %) of potentially pathogenic p53 mutations compared to ILC (13.6 %). Even though some of these potentially pathogenic mutations (4 out of 11 mutations) do not result in an amino acid change, they have been reported before in different solid tumors including breast cancer [18]. These silent mutations are of particular interest. It has already been known for decades that non-transforming mutations can affect the protein production and therefore the function by interfering with various phases of transcription and translation [29]. Examples to possible scenarios are: i**)** interference with the editing of a gene transcript if silent mutations occur in codons that contain splicing enhancers responsible for the proper removal of introns, or ii) interference with the stability of mRNA by preventing

correct folding; thus affecting the speed of translation of the protein as well as the degradation of the mRNA. Strikingly, Lamolle et al. recently showed that all reported non-transforming mutations of p53 which are documented in p53 somatic mutation database are preferentially located in conserved amino acid positions, which may depict their importance [30]. The majority of these mutations were found as single mutations, so never associated with other mutations in p53 gene, and they tended to be located inside suspected splicing enhancers. Interestingly, silent mutations observed in our study also exist as single mutations and locate either in an exposed residue of the DNA-binding domain or cause a predicted change in splicing. Moreover, silent mutations in p53 may also lead to a functional response by interfering with binding to MDM2. In this scenario, single silent point mutations in p53 mRNA can disrupt its binding to Mdm2 resulting in aberrant p53 synthesis and degradation [31]. This failure in binding is shown to reduce p53 activity, thereby supporting the notion that silent p53 mutations can affect p53 function. Therefore, we think that our observation is noteworthy and in the light of recent discoveries in this field, assumptions on non-transforming mutations should be made carefully. We have also observed two previously reported and validated polymorphisms in a high number of our cases with no significant preference for either ILC variant. Of note is the fact that these polymorphisms were shown to be related with cancer susceptibility [18]. Especially the amino acid change 72P>R (Table 3) has been shown to be associated with breast cancer susceptibility [32-36].

The observation of this significantly increased frequency of pathogenic mutations in PILC -more than half of which do probably give rise to a non-functional protein based on predictions of computer models and for some based on literature (Table 2)- is also in line with mouse studies in which stochastic somatic inactivation of p53 and E-cadherin in the mammary gland induced the development of mouse PILC [16]. For some of the observed potentially pathogenic mutations, predictions on function of the protein were not available. However, it would not be unexpected if there were activating mutations among them since mutant p53 protein can also have a distinct function in cancer such as promoting invasion and metastases [37].

Another observation in this study was the significant correlation between p53 accumulation and transforming pathogenic mutations independent of any ILC variant. The positive correlation between p53 accumulation and mutations were made by other groups [4, 38-40] although not by all [41, 42]. It is apparent that the correlation between accumulation and mutations also depends on the influence of the mutation on the half-life of the protein. Most mutant forms of p53 have a longer cellular life and are therefore recognized by antibodies while others are not. This can explain our differential observation of p53 accumulation when all mutations are counted. A mutation might cause a misfolding of the protein structure (such as the above mentioned silent mutations resting in splicing enhancers or playing a role in mRNA stability) or a truncation that affects the epitope that is recognized by the antibody. We have also observed a case with p53 accumulation but no mutations. An explanation for this may be the possibility of mutations that are located outside of the investigated exons. Interestingly, a high

percentage of p53-negative cases showed a polymorphism in residue 72, a mutation that has been implicated in enhanced targeting for degradation [43] providing a possible explanation for the p53 negativity in these cases. However, based on published data and our results, we can conclude that p53 immunostaining does not always reflect the genetic alteration and vice versa; the existence of a mutation does not always lead to p53 accumulation or complete lack of expression.

In conclusion, the clinically more aggressive pleomorphic variant of ILC bears a significantly higher frequency of p53 mutations compared to the classic variant, Moreover, since inactivation of p53 and E-cadherin in mice leads to the development of PILC, we envisage that p53 mutations may play a role in carcinogenesis of PILC.

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Chapter 7

Copy Number Analysis of Tumor Suppressor and Oncogenes in Classic and Pleomorphic Invasive Lobular Carcinoma of the Breast

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Abstract

At present, 10–15% of breast cancer patients present with invasive lobular carcinoma (ILC) which differs from ductal carcinomas in terms of biology, histology and response to therapy. The disease is clinically difficult to diagnose and has relatively poor prognosis. Even though there are crucial molecular aberrations associated with ILC (such as loss of CDH1), investigations regarding the molecular events underlying ILC development and progression is limited. Along the spectrum of ILC, several variants have been described such as the classic subtype and the rare, more aggressive pleomorphic subtype. A better understanding of the molecular pathways governing the etiology of ILC subtypes is needed to develop new diagnostic and therapeutic targets.

In order to gain more insight in the genetic profile of these two ILC subtypes, we performed multiplex ligation-dependent probe amplification (MLPA) copy number analysis for a set of established breast cancer tumor suppressors and oncogenes in 27 classic and 12 pleomorphic ILC.

Overall, we observed fewer copy number changes in classic than in pleomorphic ILC. Amplifications of FGFR1, ADAM9, PRDM14, MTDH, TRAF4 and MED1 were significantly more frequent in pleomorphic ILC. Unsupervised hierarchical clustering revealed that pleomorphic ILC clustered partly with classic ILC and partly with invasive ductal cancers.

In conclusion, copy number changes for a set of established breast cancer genes are more frequent in pleomorphic ILC than in classic ILC, compatible with their more aggressive clinical behaviour. Moreover, cluster analysis suggested that classic and pleomorphic ILC may represent separate entities, pleomorphic ILC being an entity molecularly in between classic ILC and invasive ductal cancer.

Introduction

After invasive ductal cancer (IDC), the second most prevalent histologic breast cancer type is invasive lobular breast cancer (ILC), which accounts for approximately 15% of all breast cancers [1]. ILC differs from IDC in terms of clinical presentation, biology, histology and response to therapy. Based on their molecular profile, ILCs are usually "luminal" type breast cancers expressing estrogen (ER) and molecules involved in ER activation including the progesterone receptors (PR) [2, 3]. With increasing histological grade, ILCs become resistant to endocrine therapy once the hormone receptor expression is lost [4]. As a consequence, they are less susceptible for standard chemotherapy regimens and targeted therapies against EGFR due to low/absent EGFR expression [4, 5]. In addition, there are several studies supporting that ILC has a worse prognosis compared to IDC [6]. Altogether these data underline the importance of investigations regarding the molecular events underlying lobular breast cancer development and progression.

ILC has several variants, the two most common ones being the classic and pleomorphic types. Classic ILC is composed of small regular cells with frequently intracytoplasmic vacuoles, small nuclei in a highly infiltrative growth pattern with dissociated cells and single ("indian") files, often in targetoid patterns around uninvolved ducts, and with low mitotic rate. The pleomorphic variant exhibits the growth pattern of classical ILC but with polygonal, eccentric and highly pleomorphic nuclei with nucleoli, and usually more mitoses [7]. The frequency of these subtypes among all ILC cases is suggested to be approximately 60% for classic ILC and 10% for pleomorphic ILC [8, 9]. Pleomorphic ILC has been established to have a poorer prognosis [10]. Moreover, several reports suggested that pleomorphic ILC shows similar characteristics to high grade IDC rather than to ILC [11, 12]. In general, investigations on molecular similarity and differences of classic and pleomorphic ILCs are limited which hampers the understanding of the differential progression of these subtypes. Moreover, this knowledge could provide new insight into diagnostic criteria and response to therapy of ILC patients.

So far, various oncogenes and tumor suppressors have been demonstrated to be involved in carcinogenesis, progression and therapy response of invasive breast cancers such as HER2, TOP2A, ESR1, MYC, CCDN1 and CDH1 (detailed information in Table 1). Analysis of copy number changes of these genes might be valuable for molecular profiling of different subtypes. Multiplex ligation dependent probe amplification (MLPA) is a PCR based high-throughput technique that can well be used for this purpose. With this technique, copy number of multiple genes can be determined simultaneously in a quantitative way and it is ideal when using DNA extracted from paraffin embedded material since it requires very small quantities of (fragmented) DNA. In previous studies we established the reliability and reproducibility of this technique [13, 14].

With the aim to increase the current awareness on key molecular aberrations associated genes in a group of classic and pleomorphic ILC cases. The analysis was done with the "breast cancer MLPA kit" which includes probes for ESR1, EGFR, FGFR1, ADAM9,

Gene	Chrom.Pos.	#Probes	Description of the Gene Product	Ref.
ESR1	06q25.1	2	Estrogen receptor 1; transcription factor; primarily essential for sexual development, reproductive function	[43]
EGFR	07p11.2	2	Epidermal growth factor receptor; cell surface receptor tyrosine kinase involved in signal transduction	[44]
FGFR1	08p11.23	2	Fibroblast growth factor1; receptor tyrosine kinase involved in signal transduction	[32]
ADAM9	08p11.23	1	Disintegrin and metalloproteinase domain containing protein 9; enzyme involved in cell-cell, cel-matrix interactions, protein metabolism	[32]
IKBKB	08p11.21	2	Inhibitior of kappaB kinase beta; serine/threonine kinase involved in signal transduction	[29]
PRDM14	08q13.3	1	PR domain zinc finger protein 14; involve in regulation of transcription	[45]
MTDH	08q22.1	2	Metadherin; a protein involved in invasion and metastasis	[46]
MYC	08q24.21	3	Transcription factor playing a role in proliferation and apoptosis	[47]
CCND1	11q13.2	2	G1/S-specific cyclin-D1; involved in regulation of cell cycle and signal transduction	[48]
C11orf30	11q13.5	2	Protein EMSY; involved in regulation of transcription	[48, 49]
CDH1	16q22.1	2	E-cadherin; involved in adhesion and signal transduction	[28]
TRAF4	17q11.2	1	TNF receptor-associated factor 4; involved in cell proliferation, apoptosis and signal transduction	[50]
CPD	17q11.2	1	Carboxypeptidase D; an enzyme involved in protein metabolism	[51]
MED1	17q21.2	1	Mediator of RNA polymerase II transcription subunit 1; involved in regulation of transcription	[52]
ERBB2	17q12	4	Human Epidermal growth factor Receptor 2; receptor tyrosine kinase involved in signal transduction	[15]
CDC6	17q21.2	1	Cell Division Cycle 6; involved in regulation of cell cycle and signal transduction	[37]
TOP2A	17q21.2	3	DNA topoisomerase 2-alpha; involved in controlling the topologic states of DNA during transcription	[53, 54]
MAPT	17q21.31	1	Microtubule-associated protein; involved in stabilization of microtubules	[55, 56]
BIRC5	17q25.3	3	Survivin; involved in regulation of cell cycle and apoptosis	[57]
CCNE1	19q12	2	G1/S-specific cyclin-E1; involved in regulation of cell cycle and signal transduction	[58]
AURKA	20q13.31	1	Aurora A kinase; serine/threonine kinase involved in signal transduction	[59]

Table 1. *Probes of the P078-B1 MLPA kit (MRC Holland) (modified from [15])*

IKBKB, PRDM14, MTDH, MYC, CCND1, C11orf30, CDH1, TRAF4, CPD, MED1, ERBB2, CDC6, TOP2A, MAPT, BIRC5, CCNE1 and AURKA. As described previously [15], these genes were selected based on their prognostic and/or therapeutic implications in breast cancer, or their proven frequent copy number change by comparative genomic hybridization.

Materials and Methods

Patients

Tissue samples of ILC patients were gathered as described previously [16]; and comprised archival material from 39 breast cancer patients with ILC (27 classic and 12 pleomorphic) collected from the Pathology departments of the University Medical Center Utrecht, Utrecht, and Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands, and the Institute of Pathology, Paderborn, Germany. Classic and pleomorphic subtypes were identified on Hematoxylin and Eosin (H&E) stained reference slides from formaldehyde-fixed paraffin embedded breast cancer tissue blocks of 41 cases by an experienced pathologist (PJvD), considering cases with nuclear atypia score 3 as pleomorphic ILC. Use of anonymous or coded left over material for scientific purposes is a part of the standard treatment contract with patients in our hospitals [17].

DNA extraction

After deparaffinization of the slides by standard methods, the relevant area from each slide (as identified on corresponding H&E stained sections) was scraped off with a scalpel and suspended in 50-100 µl lysis buffer (Tris-HCL pH 8.0 with 0.5 % Tween 20). After that, proteinase K (1mg/ml) was added to each sample and incubated overnight at 55°C. Next day, all the samples were incubated at 100°C for 10 minutes. Following this process the samples were centrifuged at 14000 rpm (4°C) for 30 minutes, after which the supernatant was collected and stored at -20°C.

Multiplex Ligation-Dependent Probe Amplification

MLPA was performed as described previously [15]. DNA was used for MLPA analysis according to manufacturers' instructions, using the P078-B1 kit (MRC Holland, Amsterdam, the Netherlands). Table 1 summarizes the contents of this kit. All tests were performed in duplicate by PCR (ABI 9700; Applied Biosystems, Foster City, CA, USA) and PCR products were analyzed on an ABI730 capillary sequencer (Applied Biosystems). Gene copy numbers were analyzed using Genemapper (Applied Biosystems) and Coffalyser (version 7.0) software (MRC-Holland). For genes with more than one probe present in the kit, the mean of all the probe peaks of this gene was calculated in duplicate. If the mean value was below 0.7 the respective gene was defined as lost, a value between 0.7 and ≤1.3 was defined as normal, a value between 1.3 and 2.0 as low-level amplification and values >2.0 as high-level amplified, as previously established [18, 19].

Statistics

Frequencies of copy number changes were compared between classic and pleomorphic ILC by Chi-square analysis (SPSS), grouping low and high level amplifications. Median number of copy number changes of classic and pleomorphic ILC were compared by Mann-Whitney (SPSS). P-values below 0.05 were considered significant.
Unsupervised hierarchical cluster analysis (Euclidean distance, average linkage analysis) was performed using the open-source R statistical software (http://www.r-project.org) as before [20, 21].
First, clustering was performed on the classic and pleomorphic ILC cases to identify gains and losses patterns for these entities. Thereafter, we performed a second cluster analysis including 39 IDC from a previous study analyzed with the same MLPA kit [22] to investigate whether pleomorphic ILC clusters with classic ILC or rather with IDC.

Results

Amplifications and losses in classic and pleomorphic ILC

As shown in Table 2, all analyzed genes were involved in copy number alterations, mostly as amplifications with varying frequencies. Amplifications (low and high level) were most frequently observed in genes located on chromosome 8 such as CCDN1 (11/39; 28%), MTH (10/39; 26%), and PRDM14 (10/39; 26%) and genes located in chromosome 17, such as CDC6 (12/39; 31%), and ERBB2 (8/39; 20%). The most common loss was observed in CDH1 (11/39; 28%). Losses for other genes were either not present or very rare such as AURKA (3/39; 8%), FGFR (1/39; 3%), CPD (1/39; 3%), and MAPT (1/39; 3%).

Table 2 and Figures 1 and 2 reflect the copy number changes in classic and pleomorphic cases. There was a striking difference in the frequency of amplifications and losses between these two ILC subtypes. In general, a higher frequency of low (ratio>1.3) and high level (ratio>2.0) level amplifications was observed in pleomorphic compared to classic ILC (Figure 2). Only two genes showed high level amplifications in classic ILC although rarely; FGFR (2/27; 7%) and CDC6 (1/27; 4%). In classic ILC cases, the most frequently amplified (high and low level) gene was CDC6 (7/27; 26%) followed by CCND1 (5/27; 19%). In general, the genes located on chromosome 8 (FGFR1, ADAM9, IKBKB, PRDM14, MTDH and MYC) and some of the genes located on chromosome 17 (MED1, ERBB2, TOP2A, MAPT, BIRC5) showed copy number gains (Figure1A). The same held true for the pleomorphic cases, but the frequency of amplifications (high and low) for most cases was approximately 3-4 times higher compared to the classic ILCs (Figure 1B, Figure 2), and amplification frequencies for amplified genes were mostly higher than 30%. ADAM9 (6/12; 50%), MTDH (6/12; 50%) and CCND1 (6/12; 50%), all located on chromosome 8, showed the highest amplification frequency within our study population (observed in half of the pleomorphic cases). PRDM14 and CCND1 were the genes showing most frequently high level amplification in pleomorphic cases with a frequency of 4/12 (33%).

	All cases			Classic ILC			Pleomorphic ILC			p-value*
Gene	Amp. (%)	High Level Amp. (%)	Loss (%)	Amp. (%)	High Level Amp. (%)	Loss (%)	Amp. (%)	High Level Amp. (%)	Loss (%)	
ESR1	8	0	0	11	0	0	0	0	0	0.47
EGFR	5	0	0	4	0	0	8	0	0	0.47
FGFR1	20	13	3	11	7	0	42	25	8	0.04
ADAM9	20	5	0	7	0	0	50	17	0	0.02
IKBKB	20	8	0	11	0	0	42	25	0	0.08
PRDM14	26	10	0	15	0	0	50	33	0	0.02
MTDH	26	8	0	15	0	0	50	25	0	0.02
MYC	18	5	0	11	0	0	33	17	0	0.1
CCND1	28	10	0	19	0	0	50	33	0	0.08
C11orf30	8	3	0	7	0	0	8	8	0	0.4
CDH1	0	0	28	0	0	22	0	0	41	-
TRAF4	18	0	0	7	0	0	42	0	0	0.00
CPD	0	0	3	0	0	4	17	0	0	0.08
MED1	18	5	0	7	0	0	42	17	0	0.04
ERBB2	20	8	0	11	0	0	42	25	0	0.1
CDC6	31	8	0	26	4	0	42	17	0	0.1
TOP2A	18	3	0	11	0	0	33	8	0	0.08
MAPT	20	0	3	15	0	4	33	0	0	0.1
BIRC5	20	0	0	15	0	0	33	0	0	0.1
CCNE1	3	0	0	11	0	0	8	0	0	0.3
AURKA	0	0	8	4	0	4	0	0	17	-

Table 2. F*requencies (represented as percentages) of low level amplification (ratio>1.3), high level amplification (ratio>2.0) and loss (ratio<0.7) for all 21 genes analyzed by MLPA for 39 invasive lobular breast cancer patients (27 classic and 12 pleomorphic ILC). (for references see Table1). Amp=Amplifications. * chi-square analysis classic vs. pleomorphic ILC, grouping high and low level amplifications.*

The most frequently lost region in pleomorphic ILC was CDH1 with a frequency of 5 out of 12 patients (41%); this was almost two times more frequent compared to classic cases. There were on average 6.25 amplifications per patient (range 0-17 of the 21 investigated genes) for pleomorphic ILC patients of which 2.5 were high level amplifications compared to 2 amplifications per patient for classic ILC patients of which 0.1 were high level amplifications.

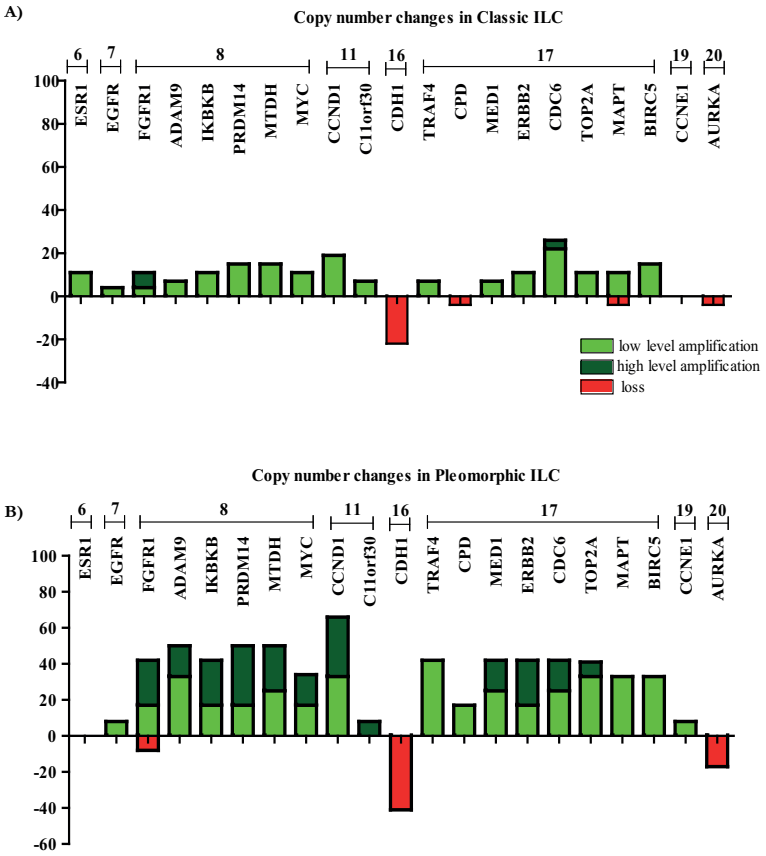

Figure1. *Copy number changes in Classic (A) and Pleomorphic (B) ILC*

Except for two classic ILC samples, the analyzed genes were never amplified or lost alone. Interestingly, 4 out of 12 (33%) pleomorphic ILC showed amplifications for all six chromosome 8 genes which were accompanied by the amplifications of some genes located on chromosome 17. This was not observed for classic cases. For one pleomorphic case (8%) both genes on chromosome 11 were amplified whereas this was the case for two patients (7%) in the classic group.

As shown in Table 2, copy number changes (lumping low and high level amplifications) were significantly different between classic and pleomorphic ILC for the genes FGFR1, ADAM9, PRDM14, MTDH, TRAF4 and MED1.

Cluster Analysis

Unsupervised hierarchical clustering analysis of classic and pleomorphic cases showed a separate gene cluster consisting of CCND1 (chromosome 11) and FGFR (chromosome 8) (Figure 3). All of the remaining chromosome 8 genes (ADAM9, IKBKB, PRDM14, MYC) were located in a second cluster and within the same subcluster.

After the analysis, there were two major clusters observed (Figure 3). The first cluster was characterized by a low frequency of gains and contained all the classic cases (twenty seven) except one and six pleomorphic cases. The second cluster was characterized by a high frequency of gains and mainly composed of pleomorphic cases.

A second clustering analysis including 39 IDC (Figure 4) revealed a separate gene cluster consisting of ERBB2, FGFR and CCND1. Reasoning from the cases, two clusters were observed. One cluster was characterized by a high frequency of copy number changes and contained mainly high grade IDC (12 grade 3, 3 grade 2 and 1 grade 1 tumor) and most pleomorphic cases.. In contrast, the second cluster was composed of classic ILC, and low-grade IDC (3 grade 1, 8 grade 2 and 12 grade 3 tumors).

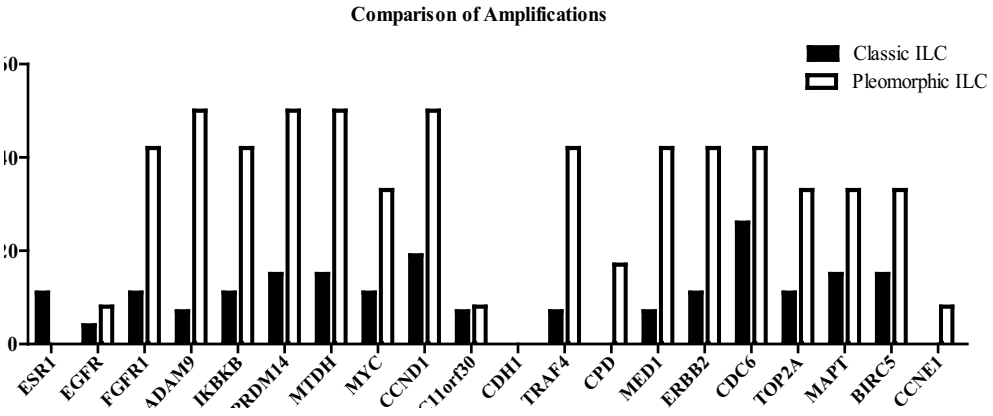

Figure2. *Comparison of amplifications between Classic and Pleomorphic ILC*

Discussion

The aim of this study was to study copy number changes of a set of established breast cancer genes associated with classic and pleomorphic ILC by MLPA analysis. Aberrant regulation of gene function through copy number changes may play a crucial role in oncogene activation and tumor suppressor gene inactivation and act as the driving force of carcinogenesis.

In our analysis, all patients showed at least one copy number change (loss or gain) representing the high genomic instability of these patients, and the suitability of this breast cancer MLPA kit. These alterations were observed in all of the analyzed genes with varying frequencies and the alterations were mostly low or high level amplifications. Losses were also observed and the most frequently lost gene was CDH1, the locus for E-cadherin [23]. Indeed, loss of E-cadherin expression by allelic loss, mutations or promoter hypermethylation is known to be the most characteristic feature of ILC [24, 25]. E-cadherin is involved in adhesion and previous research showed a strong correlation between loss of E-cadherin expression and invasion [26, 27]. Interestingly, we observed approximately two times more frequent E-cadherin loss (41%) in pleomorphic ILC compared to the classic cases (21%) supporting the recently published study of Derksen

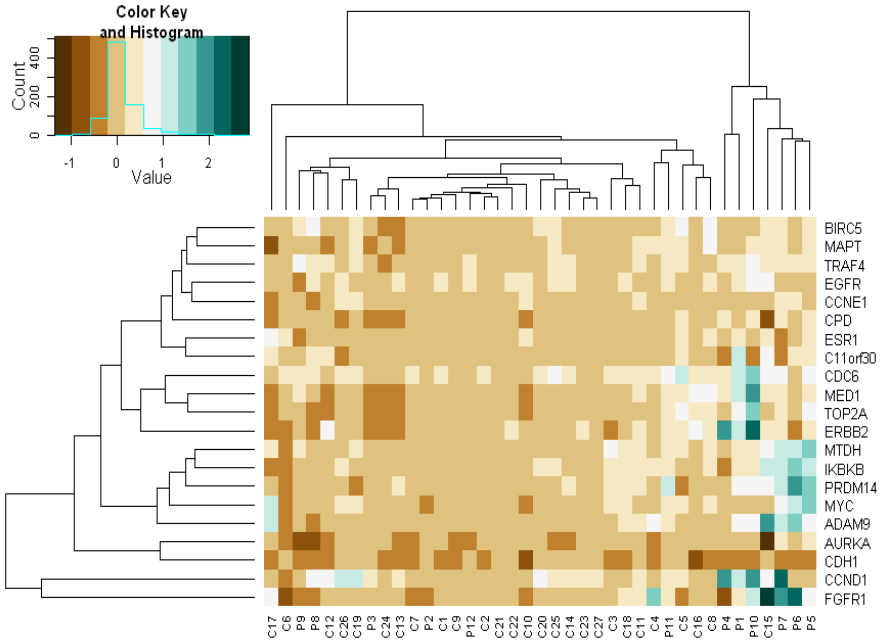

Figure 3. *Unsupervised hierarchical clustering analysis of classic and pleomorphic cases*

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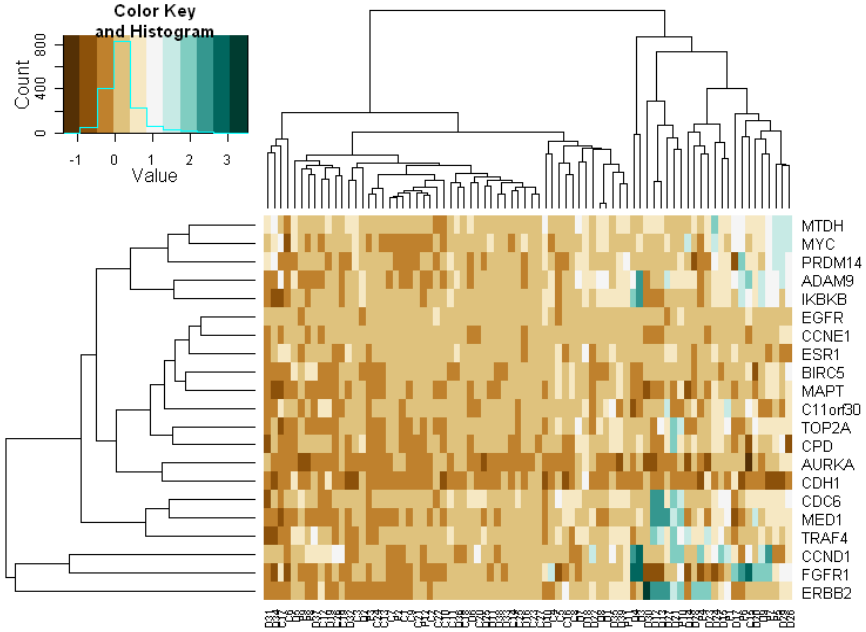

Figure 4. *Unsupervised hierarchical clustering analysis of ductal and pleomorphic ILC cases*

et al. where the authors demonstrated that somatic loss of E-cadherin together with p53 lead to human pleomorphic ILC-like tumors in mice [28].
Amplifications (low and high level) were 3-4 times more frequent in pleomorphic ILC compared to classic ILC. Half of the pleomorphic cases showed amplifications in ADAM9, MTDH and CCND1 which all locate on chromosome 8. ADAM9 is a metalloprotease involved in cell-cell, cell-matrix interactions and protein metabolism [29]. Since ADAM9 is highly expressed in cancer, including breast cancer and ADAM9 was shown to promote cancer cell migration, it is an attractive target for therapy [30]. Metadherin (MTDH) is involved in signal transduction and promotes metastases in breast cancer [31]. Moreover, its activation was associated with chemo resistance and poor prognosis [31]. The biological effect and functional importance of ADAM9 and MTDH amplifications in pleomorphic ILC cases remains to be investigated. CCND1 encodes for Cyclin D1 which plays an important role in the cell cycle, was shown to promote ER-mediated gene transcription [32] and is frequently expressed in ILC [32, 33]. It would be worthy to investigate the involvement of Cyclin D1 in the regulation of ER associated hormone sensitivity in ILC further. Another frequently amplified region in pleomorphic ILC was ERBB2, encoding for HER2. HER2 amplification and overexpression correlates with resistance to tamoxifen and conventional chemotherapy in breast cancer [34] and poor prognosis [35]. This was much lower for classic cases which supports previous reports demonstrating low/absent HER2 amplification and overexpression in classic ILC, in contrast with pleomorphic ILC [36]. CDC6, also located on chromosome 17, was shown to be frequently amplified in classic ILC. The product of CDC6 is involved in cell cycle regulation and signal transduction [37]. and upregulated in breast cancer [38]. Considering its role in cell proliferation, it stands as another interesting candidate for future investigation.
Interestingly, we observed amplifications of all the genes on the same chromosome (8 and 11) for several cases; 4 out of 12 (33%) pleomorphic ILCs showed amplifications for the six genes located on ‘chromosome 8. One pleomorphic case (8%) and two patients (7%) in the classic group showed amplifications for the two genes located on ‘chromosome 11 genes. Altogether these amplifications might be indicators of polysomy. Previously, chromosome 8 polysomy was associated with high grade [39-41] and poor prognosis of breast cancer [42]. Unsupervised hierarchical clustering analysis of 21 genes in classic and pleomorphic cases showed a separate gene cluster consisting of *CCND1* and *FGFR1*. It was also interesting to see that 50% of the pleomorphic cases formed a separate cluster, suggesting that some pleomorphic ILC might have a molecular evolution path different from classic ILC. The second cluster analysis performed including the IDC cases supported this idea since these pleomorphic ILC cases mainly clustered with high grade IDC cases. Here, *ERBB2* clustered with *CCND1* and *FGFR1*, and the frequent amplification of *ERBB2* in pleomorphic ILC cases was also similar to IDC rather than classic ILC.
In conclusion, our MLPA study has revealed interesting potential therapeutic targets in classic and pleomorphic ILC. Further, our data indicate that the more aggressive

pleomorphicILC subtype contains multiple copy number changes in different important oncogenes and tumor suppressors that differ from classical ILC. Moreover, cluster analysis demonstrated that classic and pleomorphic ILC represent separate entities which suggest that caution should be taken when defining diagnostic criteria, treatment strategies and response to therapy of patients with classic and pleomorphic ILC.

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Chapter 8

Summarizing Discussion and Future Perspectives

Breast cancer is a major concern for females as being the most frequently observed carcinoma and the most common cause of cancer related death among women (World Cancer Report, 2008). It is a histologically heterogeneous disease [1]. The second most common histologic subtype of breast cancer is lobular carcinoma of the breast [2]. Although it differs from the other subtypes in terms of molecular characteristics, a very distinguishing feature is the typical growth pattern of tumor cells in a loosely cohesive way which makes the diagnosis of this subtype of breast cancer very difficult. Certain molecular associations such as loss of adhesion molecule E-cadherin are usually linked with lobular subtype [3, 4] however a complete understanding of the contribution of different molecular pathways in initiation and progression of ILC is lacking. With the aim to unreveal some of the missing parts of this puzzle, we focused on several molecular pathways (NOTCH, p53, HIF-1α) crucial in cancer biology, in this thesis.

Lobular breast cancer

The evident simplicity of normal breast histology hides a more complex cellular composition, which becomes clear during carcinogenesis. So far, several subtypes of breast cancer have been defined, based on histology and molecular signatures. Lobular breast cancer is one of the defined histological subtypes of breast cancer and the aim of **Chapter 2** is to summarize the facts about this rare form of breast cancer by focusing on molecular and pathological features of the disease. After we introduce morphological characteristics and different subtypes of lobular breast cancer, we focus on the pathological characteristics such as diagnostic and prognostic factors.

The part dedicated on molecular characteristics is crucial since we discuss the current knowledge on the molecular pathways which are suggested to be involved in molecular evolution and progression of lobular breast cancer. This part reveals the limited knowledge on this aspect and feeds our motivation to do further research on signaling pathways involved in lobular breast cancer progression.

Mammary development and breast cancer; crucial signaling pathways

Chapter 3 provides an overview of the current literature on the role of stem cells in normal mammary gland and breast cancer development with a touch on signaling pathways involved in both processes.

A significant feature of mammary gland is the fact that the majority of its developmental changes occur after birth and this significant characteristic has allowed many researchers to address numerous fundamental questions. In this review, the evidences for the existence of mammary stem cells and tumor initiating cells are presented. Moreover, the human and mouse situation are discussed separately since there are morphological and functional differences between human and mouse mammary gland.

In the last part of the review, we comment on TGF-β, WNT, FGF Hedgehog, EGF, Estrogen and NOTCH signaling pathways (with a focus on NOTCH signaling) which are involved in mammary gland development and breast cancer formation. A better understanding of the biological roles of these pathways in normal cell behavior and

carcinogenesis may lead to discovery of novel therapies. NOTCH pathway stands out as a crucial pathway in normal breast development and differentiation. Aberrant NOTCH signaling seems to unbalance differentiation and causes excessive proliferation of cells resulting in mammary tumorigenesis. To add to this, we present novel data showing high NOTCH1 activity, a member of NOTCH family, and high expression of HES1, a common downstream target of NOTCH receptors, in normal human breast epithelium representing its importance in normal breast development.

Effect of NOTCH pathway inhibition on lobular breast cancer

Chapter 3 reveals that NOTCH signaling has not been considered before among the pathways which might play a role in lobular breast cancer development and progression. In **Chapter 4**, we investigated the outcome of NOTCH pathway inhibition on the growth of lobular breast cancer. Initially, we reported higher NOTCH activation in human invasive ILC specimen compared to the ductal cases by immunohistochemistry and this was a novel observation since so far NOTCH activation has been reported and investigated mostly in ductal breast cancer. Based on this observation, we investigated the effect of a γ-secretase inhibitor (GSI) on the proliferation of mouse and human ILC like cell lines *in vitro* and demonstrated a decrease in proliferation of these cells due to cell cycle arrest. Furthermore, we used a mouse model where orthotropic transplantation of a mouse ILC cells into mammary fat pads led to the growth of ILC like tumors. Treatment of these mice with GSI by using osmotic pumps led to NOTCH inhibition and tumor retardation *in vivo*.

HIF-1a and NOTCH signaling in ductal and lobular breast cancer

NOTCH signaling has been shown to interplay with many different pathways (Chapter3 and Chapter 4) and recently, several different studies indicated a link between hypoxia inducible factors (HIFs) and NOTCH activation in different contexts and tissues resulting in tumorigenesis. In **Chapter 5,** we investigated if such an association exists in primary and metastatic human breast cancer specimen and if it has any prognostic effect. For this purpose, firstly, we checked the expression of HIF-1α and NOTCH1 intracellular domain (N1ICD; active NOTCH1) and their known downstream targets in a group of patient material. Even though expressions of both proteins correlated with their downstream targets, there was no association between HIF-1α and NOTCH1 signaling. Moreover, co-expression of N1ICD and HIF-1α was significantly associated with high tumor grade and high mitotic index but had by itself no prognostic value. During this study, we have also made some independent but appealing observations. For example, pleomorphic lobular cancers had more often HIF-1α expression in tumor cells and stroma compared to classic lobular cancers.

Interestingly, primary breast cancers expressed higher levels of N1ICD than their corresponding metastases, especially in the cases which metastasized to brain and to skin and NOTCH pathway activation reflected worse prognosis. However, in short NOTCH and HIF signaling do not seem to be functionally associated in breast cancer.

Frequency of p53 mutations in classic and pleomorphic lobular breast cancer

Invasive lobular breast cancer does also have variants such as the most commonly seen classic and pleomorphic variants. Although these two variants exhibit the same growth pattern, the pleomorphic variant is described with more aggressive clinical behavior. Recently, our group reported the development of pleomophic ILC (PILC) like mammary tumors in conditional mouse models where p53 and E-cadherin is inactived. This interesting observation led to **Chapter 6** which reports our investigation of p53 mutations and p53 accumulation in a group of human classic and PILC cases by direct DNA sequencing and immunohistochemistry. Athough we did not observe a significant difference in p53 protein accumulation between the pleomorphic and classic variant, a higher number of potentially pathogenic mutations in PILC cases were detected compared to the classic variant. Together with the observation of p53 and E-cadherin inactivation leading to PILC like phenotype in mice, p53 mutations might play a crucial role in PILC carcinogenesis.

Copy number changes in tumor suppressor and oncogenes in classic and pleomorphic ILC

A variety of oncogenes and tumor suppressors have been demonstrated to be involved in carcinogenesis, progression and therapy response of invasive breast cancers. Analysis of copy number changes of these genes might be valuable for molecular profiling of different subtypes, and thus individual patients suffering from them. In Chapter 7, we used Multiplex ligation dependent probe amplification (MLPA) with the aim to gain a better idea on the genetic profile of classic and pleomorphic ILC subtypes which could ultimately lead to novel diagnostic and therapeutic targets for these ILC subtypes. We reported that copy number changes for a set of established breast cancer genes are more frequent in pleomorphic ILC than in classic ILC, compatible with their more aggressive clinical behavior. Moreover, cluster analysis demonstrated classic and pleomorphic ILC to be rather separate entities and pleomorphic ILC as an entity molecularly in between classic ILC and infiltrating ductal cancer.

Future Perspectives

Over the last decades, investigations on molecular features which are characteristic to certain subtypes of breast cancer has led to increased understanding to molecular evolution and progression of different subtypes while increasing future targets for prevention and treatment. Invasive lobular breast cancer is the second most prevalent type of breast cancer and there is a strong need for a better understanding of ILC to improve treatment. The rationale behind our investigations in this thesis is to gain new insight into ILC and different variants of it. This is necessary since the diagnosis of ILC is difficult and overall ILC has a worse prognosis compared to invasive ductal cancer (IDC) reflecting the low success rate of current treatment strategies against ILC.

The rapidly emerging mammary stem cell (MaSC) field allows us to have awareness on the existence of these cells and the signaling pathways regulating them. Evidently, there is a delicate balance between homeostasis and deregulation of these pathways leading

to breast cancer. There are still crucial unanswered questions left in this field such as the differences between mouse and human mammary gland. Considering the amount of research performed by using mouse mammary gland as model, we believe that the differences between human and mouse mammary gland should be investigated further to shed light on the conclusions driven based on these studies. Besides, focusing on the nature of interactions between MaSCs and the niche within the mammary gland will provide further knowledge on signaling pathways involved in normal stem cells homeostasis and transformation into cancer stem cells. NOTCH signaling is believed to be one of these pathways as NOTCH proteins are involved in various aspects of cell fate determination in mammary gland [5]. This pathway has been demonstrated to be involved carcinogenesis in different contexts and also in (ductal) breast cancer.

Our findings show that NOTCH signaling is likely to be crucial for invasive lobular breast cancer (ILC) etiology and that NOTCH inhibition using GSI may provide additional treatment options in the management of ILC. The most relevant two questions for further investigation would be; 1) what is the cause behind high NOTCH activation in ILC; 2) can we confirm the anti-tumor effect of GSI with a genetic approach? The reasons behind our observation of high NOTCH activation in ILC still need to be determined. To be able to answer this first question, one would need to look into multiple processes controlling NOTCH activation, such as, proteolysis, glycosylation, ubiquitylation and phosphorylation. Overexpression of ligands or loss of negative regulators and/or mutations in the regulatory units of pathway members should be investigated. Moreover, NOTCH activation can be triggered by other oncogenic pathways [5]. Moreover, to confirm our demonstration of the anti-tumor effects of GSI treatment on in vivo ILC model, following a genetic approach such as making use of lobular breast cancer cell lines where NOTCH signaling is genetically blocked could be an option.

The HIF signaling pathway is another highly conserved pathway through evolution, and plays a role in different cellular processes [6, 7]. HIFs and NOTCH signaling have various overlapping effects in normal development and various cancers [8-10]. Interestingly, hypoxia is suggested to stimulate NOTCH signaling via HIF transcription factors. Although we did not observe a significant association of HIF-1α and N1ICD expression in our group of human breast cancer cases, we did have several independent interesting observations with a potential for future research. Firstly, our observation of the prognostic value of HES1 is encouraging and the prognostic value of other NOTCH targets should be investigated further since they may have the potential as prognostic marker in breast cancer. Moreover, high HIF-1α expression is in pleomorphic lobular cancers, which also frequently show stromal HIF-1α expression, might have value to clarify the more aggressive clinical behavior of this specific subtype. Because it has been speculated that HIF-1α might have a tumor promoting role in breast cancer associated fibroblasts. Novel therapies targeting HIF-1α may add to the current treatment strategies of pleomorphic cases.

Gaining understanding on the molecular changes in major signaling pathways in different

subtypes of ILC holds potential to reveal targets as a tool for differential diagnosis and treatment of classic ILC versus pleomorphic variant. The *TP53* tumor suppressor gene has been an interesting target to investigate in ILC since it is very frequently altered in other human cancers including breast cancer [11]. According to our finding PILC bears higher frequency of p53 mutations. Although some of these mutations were reported before in other cancer types, predictions on function of the protein are not available. It would be very interesting to investigate the functional effect of these mutations, since mutant p53 protein can also have very distinct functions in cancer such as promoting invasion and metastases. This would give more insight on carcinogenesis of PILC.

The copy number analysis by using breast cancer dedicated MLPA kit has revealed interesting and differential carcinogenetic and therapeutic targetsfor future investigations in classic and pleomorphic ILC. Several genes as FGFR1, ADAM9, PRDM14, MTDH, TRAF4 and MED1 showed significantly higher levels of amplifications in pleomorphic cases. Especially ADAM9 and MTDH amplifications were observed in 50% of all pleomorphic ILC patients, which is in line with the more aggressive phenotype of this subtype since both ADAM9 and MTDH were suggested to play role in invasion and metastases [12, 13]. Moreover, CDC6 was shown to be frequently amplified in classic ILC. Considering reports suggesting CDC6 enhances cell proliferation in breast cancer, it stands as another interesting candidate for future investigation [14, 15]. The fact that all patients showed at least one copy number change (loss or gain) representing the high genomic instability of these patients, and the suitability of this breast cancer MLPA kit. It was also interesting to observe that cluster analysis demonstrated classic and pleomorphic ILC to be rather separate entities and pleomorphic ILC as an entity molecularly in between classic ILC and infiltrating ductal cancer. This observation reveals that caution should be taken when defining diagnostic criteria, treatment strategies and response to therapy of patients with classic and pleomorphic ILC.

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Chapter 9

IA) Nederlandse Samenvatting - IB) Türkçe Özet

II) Acknowledgements / Teşekkür

IIIA) Curriculum vitae - IIIB) Özgeçmiş

IV) Publication List / Yayınlar

IA) Nederlandse Samenvatting

Borstkanker is onder vrouwen in zowel de westerse als niet-westerse wereld de meest voorkomende kankergerelateerde doodsoorzaak (World Cancer Report, 2008). Ongeveer 1 op de 8 vrouwen in de westerse wereld krijgt borstkanker, en er komen wereldwijd per jaar meer dan 1 miljoen nieuwe gevallen bij, waarvan 35% uiteindelijk aan kanker zal overlijden. Borstkanker is een heterogene en complexe ziekte met verschillende histologische en moleculaire karakteristieken. Invasief ductaal carcinoom (IDC) is veruit de meest voorkomende vorm van borstkanker (~80%). De overige gevallen van borstkanker bestaan uit invasief lobulair carcinoom (ILC; 10-15%) en zeldzame subtypes. Lobulaire en ductale carcinomen hebben verschillende moleculaire en histologische eigenschappen en therapierespons. Een typische eigenschap van lobulaire borstkanker is het karakteristieke groeipatroon van de tumorcellen: ze groeien heel diffuus als strengen en losse cellen, hetgeen de diagnose van dit subtype bemoeilijkt. Bepaalde moleculaire fenotypen, zoals verlies van het adhesiemolecuul E-cadherine, worden vaak geassocieerd met het lobulaire subtype, maar het is nog niet volledig duidelijk hoe verschillende moleculaire processen bijdragen aan de initiatie en progressie van ILC. Om hier verder inzicht in te krijgen, bestuderen we in dit proefschrift een aantal moleculaire processen (NOTCH, p53, HIF-1α) die een belangrijke rol spelen in de kankerbiologie.

Lobulaire borstkanker

Hoewel de histologie van de normale borst simpel lijkt, gaat er een complexe cellulaire compositie achter verscholen, die pas bij carcinogenese duidelijk wordt. Tot nu toe zijn er op basis van histologische en moleculaire kenmerken verschillende subtypes van borstkanker gedefinieerd. Lobulaire borstkanker is een van de gedefinieerde histologische subtypes van borstkanker, en het doel van **Hoofdstuk 2** is om de feiten van deze zeldzame vorm van borstkanker samen te vatten met de focus op de moleculaire en pathologische eigenschappen van deze ziekte. Nadat we de morfologische eigenschappen en verschillende subtypes van lobulaire borstkanker geïntroduceerd hebben, concentreren we ons op de pathologische karakteristieken van lobulaire borstkanker, zoals diagnostische en prognostische factoren.
Deze analyse van de moleculaire karakteristieken is cruciaal, omdat we de bestaande kennis van de moleculaire processen bespreken die een rol kunnen spelen in de moleculaire evolutie en progressie van lobulaire borstkanker. Dit gedeelte toont de beperkte kennis op dit gebied en heeft ons er toe gedreven verder onderzoek te doen naar de signaleringsroutes betrokken bij de progressie van lobulaire borstkanker.

Borstontwikkeling en borstkanker; cruciale signaleringsroutes

Hoofdstuk 3 geeft een overzicht van de bestaande literatuur over de rol van stamcellen in de normale ontwikkeling van de borstklier en borstkanker, en gaat kort in op de signaleringsroutes betrokken bij beide processen.
Een belangrijke eigenschap van de borstklier is dat de ontwikkeling voornamelijk plaats

vindt na de geboorte. Dit heeft veel onderzoekers de mogelijkheid gegeven om een aantal fundamentele vraagstukken te beantwoorden. In dit overzicht wordt het bewijs voor het bestaan van borst- en tumorstamcellen uiteengezet. Verder wordt de situatie in mensen en muizen apart besproken, aangezien er morfologische en functionele verschillen zijn in de borstklier van mens en muis.
In het laatste deel van dit overzicht bespreken we de TGF-β, WNT, FGF, Hedgehog, EGF, oestrogeen, en NOTCH signaleringsroutes (met een focus op NOTCH signalering) die betrokken zijn bij borstklierontwikkeling en borstkanker vorming. Een beter begrip van de biologische rollen van deze routes in normaal celgedrag en carcinogenese kan leiden tot de ontdekking van nieuwe therapieën. De NOTCH route is een uitzonderlijk belangrijke route in de normale ontwikkeling en differentiatie van de borst. Afwijkende NOTCH signalering lijkt de balans van differentiatie te verstoren, met als gevolg excessieve proliferatie resulterend in het ontstaan van borstkanker. Ook presenteren we nieuwe data die duiden op een hoge activiteit van NOTCH1, een lid van de NOTCH familie, en op een hoge expressie van HES1, een effector eiwit van NOTCH receptoren, in normaal humaan borstepitheel. Dit onderstreept het belang van NOTCH in de normale ontwikkeling van de borst.

Het effect van de remming van NOTCH signalering op lobulaire borstkanker

Hoofdstuk 3 laat zien dat de rol van NOTCH signalering in de ontwikkeling en progressie van lobulaire borstkanker nog niet eerder bestudeerd is.

In **Hoofdstuk 4** hebben we onderzocht wat het effect is van de remming van de NOTCH route op de groei van lobulaire borstkanker. Eerder hebben we al met immunohistochemie laten zien dat er hogere NOTCH activatie is in gevallen van humaan ILC in vergelijking met ductale gevallen van borstkanker. Dit was een nieuwe observatie, aangezien NOTCH activatie tot nu toe vooral beschreven en bestudeerd was in ductale borstkanker. Gebaseerd op deze observatie hebben we gekeken wat het effect was van een γ-secretase remmer (GSI) op de proliferatie van muizen en humane ILC-achtige cellijnen *in vitro*. Deze bleek geremd te worden als gevolg van een blokkering in de celcyclus. Tevens hebben we een muismodel gebruikt waarin de orthotope transplantatie van muizen ILC cellen in de borstklier leidde tot de groei van ILC-achtige tumoren. Behandeling van deze muizen met GSI via osmotische pompjes leidde tot NOTCH remming en inhibitie van tumorgroei *in vivo*.

HIF-1α en NOTCH signalering in ductale en lobulaire borstkanker

Het is bekend dat NOTCH signalering een rol speelt in veel verschillende routes (Hoofdstuk 3 en Hoofdstuk 4). Recentelijk hebben verschillende studies een link gesuggereerd tussen hypoxia-geinduceerde factoren (HIFs) en NOTCH activatie in verschillende contexten en weefsels, resulterend in tumorgenese. In **Hoofdstuk 5** bestuderen we of een dergelijke link bestaat in weefsels van primaire humane borstkanker en metastases, en of deze link enige prognostische waarde heeft. Hiervoor controleerden we eerst wat de expressie niveaus waren van HIF-1α en het NOTCH1

intracellulaire domein (N1ICD; actief NOTCH1) en hun bekende effector eiwitten. Hoewel de expressie niveaus van beide eiwitten correleerden met hun effector eiwitten, was er geen associatie tussen HIF-1α en NOTCH1 signalering. Verder was er een significante associatie tussen de co-expressie van N1ICD en HIF-1α en een hoge tumorgraad en mitotische index. Er was echter geen prognostische waarde van de co-expressie van N1ICD en HIF-1α op zich. Tijdens deze studie hebben we ook een aantal onafhankelijke maar interessante observaties gedaan. Zo hadden pleomorfe lobulaire carcinomen vaker HIF-1α expressie in tumorcellen en stroma dan klassieke lobulaire kankers.
Primaire borstkankers brachten hogere niveaus van N1ICD tot expressie dan hun bijbehorende metastasen, vooral in gevallen van hersen- en huidmetastasen, en NOTCH activatie duidde op een slechtere prognose. Samenvattend lijkt het er echter op dat NOTCH en HIF signalering niet functioneel met elkaar verbonden zijn in borstkanker.

Frequentie van p53 mutaties in klassieke en pleomorfe lobulaire borstkanker

De meest voorkomende varianten van invasieve lobulaire borstkanker zijn de klassieke en pleomorfe subtypes. Hoewel deze twee varianten hetzelfde groeipatroon vertonen, gedraagt de pleomorfe variant zich in klinisch opzicht vaak agressiever. Recentelijk heeft onze groep aangetoond dat pleomorfe ILC (PILC)-actige borsttumoren ontwikkelen in muismodellen waarin p53 en E-cadherine conditioneel afwezig zijn. Deze interessante observatie heeft geleid tot **Hoofdstuk 6**, waarin we p53 mutaties en p53 accumulatie bestuderen in een groep van humane klassieke en pleomorfe ILC gevallen via het direct sequencen van het DNA en immunohistochemie. We vonden geen significant verschil in p53 eiwit accumulatie tussen de pleomorfe en klassieke variant van ILC, maar vonden wel een hoger aantal van potentieel pathogene mutaties in PILC gevallen ten opzichte van de klassieke variant. Samen met onze observatie dat het ontbreken van p53 en E-cadherine leidt tot een PILC-achtige fenotype in muizen, suggereert dit dat p53 mutaties een belangrijke rol kunnen spelen in PILC carcinogenese.

Genkopie aantallen van tumorsuppressor en oncogenen in klassieke en pleomorfe ILC

Verschillende oncogenen en tumorsuppressor genen spelen een rol in carcinogenese, progressie en therapierespons in invasieve borstkanker. Het bepalen van genkopie aantallen van deze genen kan helpen om verschillende subtypes moleculair te classificeren en daarmee van waarde zijn voor de individuele patiënt. In **Hoofdstuk 7** gebruiken we de techniek multiplex ligation dependent probe amplification (MLPA) om meer inzicht te krijgen in het genetische profiel van klassieke en pleomorfe ILC. Een beter begrip hiervan zou uiteindelijk kunnen leiden tot nieuwe diagnostische en therapeutische aanknopingspunten. We laten zien dat de genkopie aantallen van een bepaalde set borstkankergenen vaker afwijkend zijn in de pleomorfe vorm van borstkanker vergeleken met de klassieke vorm. Dit komt overeen met het feit dat de pleomorfe vorm klinisch ook agressiever is. Bovendien liet cluster analyse zien dat de

pleomorfe en de klassieke vorm aparte entiteiten zijn. Pleomorfe ILC blijkt moleculair een aparte entiteit te zijn tussen klassieke ILC en invasief ductaal carcinoom.

Perspectieven

De afgelopen decennia is er steeds meer bekend geworden over de moleculaire kenmerken van de verschillende subtypes van borstkanker. Dit heeft geleid tot een beter begrip van hoe de verschillende subtypes moleculair gezien zijn ontstaan en zich ontwikkelen, maar ook tot nieuwe aanknopingspunten voor preventie en therapie. Invasief lobulair carcinoom is de op een na meest voorkomende vorm van borstkanker. Het is belangrijk het ontstaansmechanisme van ILC te begrijpen, zodat betere behandeling mogelijk is. De beweegreden van het beschreven onderzoek in dit proefschrift is dan ook om ILC en de verschillende subtypes beter te begrijpen. De diagnose van ILC is namelijk lastig en deze vorm van borstkanker heeft, door het lage succes van de huidige behandelingen, een slechtere prognose dan de invasief ductale vorm.

De kennis over stamcellen en de bijbehorende signaleringsroutes in de borst ontwikkelt zich snel. Er is een dunne lijn tussen de normale homeostase van deze signaleringsroutes en deregulatie hiervan, die kan leiden tot het ontstaan van borstkanker. Het antwoord op belangrijke vragen in dit veld blijven echter nog onbeantwoord, zoals de verschillen tussen de borstklier van de muis en de mens. Het merendeel van het onderzoek wordt uitgevoerd in muizen. Daarom vinden wij het van belang deze verschillen verder te bepalen, voordat definitieve conclusies worden getrokken uit het onderzoek bij muizen. Daarnaast zal het onderzoek naar de interacties tussen de stamcellen in de borst en de niche waarin de stamcellen zich bevinden meer kennis geven over de betrokken signaleringsroutes bij de transformatie van normale stamcellen naar kankerstamcellen. NOTCH signalering wordt gezien als een van deze signaleringsroutes, omdat NOTCH eiwitten betrokken zijn bij de differentiatie van de cellen in de borstklier. NOTCH signalering is betrokken bij verschillende vormen van kanker, waaronder ook (ductale) borstkanker.

Onze bevindingen laten zien dat NOTCH signalering waarschijnlijk cruciaal is voor het ontstaan van ILC. Bovendien kan het remmen van NOTCH met GSI een nieuwe therapeutische optie zijn in de behandeling van ILC. De twee belangrijkste vragen om te beantwoorden in verder onderzoek zijn; 1) wat is de oorzaak van de hoge mate van activering van NOTCH in ILC; 2) kunnen we het effect van GSI op tumorgroei bevestigen met een genetische benadering? De oorzaak van de hoge NOTCH activering die wij in ILC zien, moet nog nader onderzocht worden. Om deze eerste vraag te beantwoorden zal gekeken moeten worden naar de verschillende manieren waarop NOTCH activering gereguleerd wordt, zoals proteolyse, glycosylering, ubiquitylering and fosforylering. Overexpressie van interactiepartners of negatieve regulatoren en/of mutaties in de regulerende onderdelen van deze signaleringsroutes moeten onderzocht worden. Daarnaast kan NOTCH geactiveerd worden door andere oncogene signaleringsroutes. Om onze bevinding dat GSI een negatief effect heeft op tumorgroei in een *in vivo* ILC model te verifiëren, kan gebruik gemaakt worden van bijvoorbeeld lobulaire borstkanker

cellijnen waar de signalering van NOTCH genetisch is geblokkeerd.
Een andere belangrijke route is de signalering via HIF. De HIF signaleringsroute speelt een rol in verschillende processen in de cel en is sterk geconserveerd tijdens evolutie. HIF en NOTCH hebben een overlappende rol in de normale ontwikkeling en ook bij de vorming van kanker. Een interessant concept is dat hypoxie zou kunnen leiden tot de stimulering van NOTCH via HIF transcriptiefactoren. Hoewel wij geen significante associatie vonden tussen HIF-1α en N1ICD expressie in onze groep van humane borstkankergevallen, waren er andere observaties die verder onderzocht kunnen worden. Ten eerste is de prognostische waarde van HES1 bemoedigend en ook de prognostische waarde van andere NOTCH effector eiwitten zou onderzocht moeten worden. Bovendien kan de hoge expressie van HIF-1α in pleomorfe ILC gevallen, die ook vaak HIF-1α expressie in het stroma laten zien, het meer agressieve gedrag van dit subtype verklaren. HIF-1α speelt namelijk mogelijk een rol in het stimuleren van tumorgroei door borstkanker geassocieerde fibroblasten. Het beïnvloeden van HIF-1α kan een extra toevoeging zijn aan de huidige behandeling van pleomorfe ILC.
Het beter begrijpen van de moleculaire veranderingen in de belangrijke signaleringsroutes in de verschillende subtypes van ILC kan leiden tot nieuwe aanknopingspunten in de diagnose en behandeling van de klassieke versus de pleomorfe vorm. Het tumorsuppressorgen *TP53* is interessant om nader te onderzoeken in ILC, aangezien dit gen vaak is veranderd in kanker, waaronder borstkanker. Wij tonen aan dat p53 in PILC vaker is gemuteerd. Enkele van deze mutaties waren al bekend in de literatuur bij andere vormen van kanker, maar het effect op de functie van het eiwit niet. Het is belangrijk de functionele effecten van deze mutaties te bepalen, omdat een gemuteerd p53 eiwit verschillende effecten kan hebben in kanker zoals het stimuleren van invasie en metastasering. Meer kennis hierover zal inzicht geven in de carcinogenese van PILC.
Het bepalen van genkopie aantallen met behulp van een speciale MLPA kit voor borstkanker toont interessante aanknopingspunten voor toekomstige studies naar carcinogenese and therapeutische mogelijkheden. In pleomorfe gevallen waren genen zoals *FGFR1, ADAM9, PRDM14, MTDG, TRAF4* en *MED1* vaker geamplificeerd. *ADAM9* en *MTDH* waren in 50% van de pleomorfe gevallen geamplificeerd wat overeenkomt met het meer agressieve fenotype van dit subtype. Zowel *ADAM9* als *MTDH* zouden namelijk een rol spelen in invasie en metastasering. *CDC6* was vaak geamplificeerd in het klassieke subtype. Literatuur laat zien dat *CDC6* mogelijk een rol speelt in celproliferatie in borstkanker en hiermee is *CDC6* een andere kandidaat voor verder onderzoek. Alle onderzochte patiënten toonden ten minste één verandering in genkopie aantallen (amplificatie of verlies), overeenkomstig met de genetische instabiliteit in deze patiënten, en aangevend dat deze borstkanker MPLA kit goed werkt.
Het is interessant dat bij cluster analyse klassieke en pleomorfe ILC aparte entiteiten blijken. Het pleomorfe subtype is moleculair een aparte entiteit en bevindt zich tussen klassieke ILC en invasief ductaal carcinoom.

IB) Türkçe Özet

Meme kanseri, hem gelişmiş hem de gelişmekte olan ülkelerde, kadınlarda kansere bağlı ölümlerin en sık nedenidir (Dünya Kanser Raporu, 2008). Ortalama olarak her 8 kadından biri meme kanserine yakalanma riski ile karşı karşıyadır. Bu rakam her yıl dünya çapında bir milyondan fazla yeni vaka demektir. Meme kanserine yakalanan kadınların %35'i hayatını kaybeder. Histolojik ve moleküler açıdan çok farklı tümor çeşitlerini içinde barındıran bu kanser türü, heterojen ve komplike bir hastalıktır. En sık gorülen meme kanseri çeşidi olan invaziv duktal (IDC) meme kanserine, hastaların %80 ininde rastlanır. Invaziv lobüler meme kanseri (ILC) ise %10-15'lik bir kesimde görülür, geriye kalan hastalarda daha nadir meme kanseri çeşitlerine rastlanır. Lobüler ve duktal meme kanseri, moleküler özellikleri, histolojileri ve tedaviye yanıtları açısından birbirinden farklıdır. Lobüler meme kanserinin ayırt edici özelliği olan tümör hücrelerinin birbirinden bağımsız biçimde büyümesi, bu tipin tanısını güç hale getirir. Lobüler meme kanseri, adezyon molekülu E-kaderinin kaybı gibi bazı tipik moleküler özellikler göstersede, bu tipin inisiyasyonu ve ilerlemesine hangi moleküler yolların katkı sağladığı kesin değildir. Bu tezde, bu bulmacanın bazı eksik parçalarını çözme amacı ile, kanser biyolojisinde önemli yeri olan, birkaç farklı moleküler sinyal yolu (NOTCH, p53, HIF-1α) üzerinde durulmuştur.

Lobüler meme kanseri

Normal meme histolojisinin ilk bakışta görülen sadeliği daha karmaşık bir hücresel kompozisyonu gizlemektedir. Bu karmaşık kompozisyon daha çok karsinogenez sırasında açığa çıkar. Günümüzde, meme kanserinin histolojik ve moleküler açıdan birçok farklı çeşidi olduğu bilinmektedir. Lobüler meme kanseri de tanımlanan bu histolojik çeşitlerden biridir. Bu tezin 2. bölümünde, meme kanserinin bu nadir formunun bilinen moleküler ve patolojik özellikleri özetlenmektir. Bu bölümde ayrıca, hastalığın morfolojik özellikleri ve farklı alt çeşitlerinin yanı sıra tanısal ve prognostik faktörler gibi patolojik özelliklerine de yer verilmiştir. Lobüler meme kanserinin moleküler evrim ve gelişme sürecine dahil oldugu varsayılan moleküler sinyal yolları hakkında elde ettiğimiz sınırlı bilgi, bu konuda daha fazla araştırma yapmak için bizi motive etmektedir.

Meme gelişimi ve meme kanseri; önemli sinyal yolları

Bölüm 3'de; kök hücrelerinin normal meme gelişimi ve kanser inisiasyonundaki rolü ve bunda rol oynayan sinyal yolları ile ilgili güncel literatüre dayanan genel bir özet yer almaktadır.

Meme bezinin önemli bir özelliği, gelişimsel değişikliklerin çoğunun doğumdan sonra meydana gelmesidir ve bu önemli özellik birçok araştırmacının meme bezi ile ilgili temel soruları cevaplamasını sağlamıştır.

Bölüm 3 de yer alan derlemede, meme kök hücrelerinin varlığına dair kanıtlar sunulmuştur. Insan ve fare meme bezi arasında morfolojik ve fonksiyonel farklılıklar bulunması nedeniyle, bu iki model ayrı ayrı ele alınmıştır. Derlemenin son bölümünde, meme bezi gelişimi ve meme kanseri oluşumunda rolü olan TGF-β, WNT, FGF, Hedgehog, EGF,

Östrojen ve NOTCH sinyal yolları hakkında bilgi verilmiştir. Bu sinyal yollarının normal hücre davranışı ve karsinogenezdeki rollerinin daha iyi anlaşılması, yeni tedavi yollarının geliştirilmesine yardımcı olabilir. Ozellikle NOTCH sinyal yolu, normal meme gelişimi ve hücre farklılaşmasında büyük rol oynamaktadır. Bu sinyal yolunda meydana gelen bozukluklar, dengesiz hücre farklılaşması ve tümör oluşumuna sebebiyet verir.
Bu bölümde, normal meme epitelinde NOTCH ailesinin bir üyesi olan NOTCH1'in yüksek aktivitesini gösteren yeni verilere yer verilmiştir.

NOTCH sinyal yolu inhibisyonunun lobüler meme kanseri üzerindeki etkisi

4. bölümde NOTCH sinyal yolu inhibisyonunun, lobüler meme kanseri büyümesi uzerindeki sonuçları araştırılmıştır. Immünohistokimyasal deney sonuçlarımızda, ILC örneklerinde yüksek NOTCH aktivasyonuna rastlanmıştır. Literatürde yüksek NOTCH aktivasyonu genelde IDC örneklerinde rapor edildiği için, ILC deki bu durum yeni bir gözlemdir. Bu gözleme dayanarak, NOTCH sinyal yolunu bloke eden bir kimyasal olan γ-sekretaz inhibitörü (GSI) kullanılmıştır. Fare ve insan ILC hücreleri kullanılarak yapılan in vitro ve in vivo deneylerde bu inhibitor hücre döngüsünün durmasına sebebiyet vermiş ve tümör hücrelerinin bölünmesinde bir azalma gözlemlenmiştir.

HIF-1α ve NOTCH sinyal yollarının duktal ve lobüler meme kanserindeki yeri

Birbirinden ayrı yapılmış birkaç çalışmada, farklı dokuların normal gelişiminde ve tümörogenezinde hipoksi ile indüklenebilir faktörler (HIFs) ve NOTCH sinyal yolunun birbiriyle etkileşimi rapor edilmiştir. Bu tezin 5. bölümünde, bizde bu etkileşimi bir grup primer ve metastatic hasta örneğinde inceledik. HIF-1α ve NOTCH1 aktif formunun birlikte ekspresyonu tümörlerde yüksek grade ve yüksek proliferasyonla ilişkili bulunmuştur. Yüksek HIF-1α ekspresyonu pleomorfik lobüler meme kanserinde öne çıkmıştır. NOTCH ve HIF sinyali yolları fonksiyonel olarak ilişkili görünmüyorsa da, NOTCH sinyal yolu aktivasyonu kötü prognozla bağlantılı bulunmuştur.

Klasik ve pleomorfik lobüler meme kanseri TP53 mutasyonlarının sıklığı

İnvaziv lobüler meme kanserinin sık görülen iki türevi bulunmaktadır; klasik ve pleomorfik. Bu iki varyant aynı büyüme paterni ortaya koymasına rağmen, pleomorfik varyant klinik açıdan daha agresif davranış sergiler. Yakın zamanda, fare modellerinde p53 ve E-cadherin inaktivasyonunun pleomorfik ILC ine benzer meme tümörlerine yol açtığı rapor edilmiştir. Bu ilginç gözlemle birlikte, bölüm 6 da bahsedilen DNA analizi ve immünohistokimyasal yöntemlere dayanarak, klasik ve pleomorfik ILC örneklerinde, bir tümör baskılayıcı olan p53 genindeki mutasyonları ve p53 protein birikimini inceledik. Bu inceleme sonunda daha agresif olan pleomorfik varyantlarda, daha fazla p53 mutasyonu gözlemlendi. Bu sonuçlar, PILC modeli oluşturulan farelerde elde edilen bu sonuçlarla beraber, p53'ün PILC karsinogenezinde önemli bir rol oynadığı gösterilmiştir.

Klasik ve pleomorfik ILCde tümör baskılayıcı genler ve onkogenler, DNA kopya sayısı değişiklikleri

Birçok tümör baskılayıcı gen ve onkogenin invaziv meme kanserlerinin gelişimi,

ilerleme ve tedaviye yanıt şeklinde etkisi olduğu gösterilmiştir. Bu genlerin DNA kopya sayılarındaki değişiklikleri farklı kanser tiplerinde incelemek, bu kanser tiplerinin görüldüğü hastalara bireysel açıdan daha iyi teşhis konması ve tedavi uygulanması demektir. Bölüm 7'de, klasik ve pleomorfik ILC alt tiplerinin genetik profili üzerine daha iyi bir fikir elde etmek amacı ile Multiplex ligasyonu bağımlı prob amplifikasyonu (MPLA) tekniğini kullanarak, bilinen bazi tümör baskılayıcı ve onkogenlerin DNA kopya sayısındaki değişiklikleri rapor ettik. Genel olarak pleomorfik ILC örneklerinde daha fazla kopya sayısı değişiklikleri gözlemlenmistir.

Sonuç olarak, bu araştırma invaziv lobuler meme kanserinin hem oluşma hem de ilerleme aşamasına dair anlayışı geliştirmiş ve bu kanserin görüldüğü hastalarda hipoksi ve NOTCH sinyal yollarını kullanarak bireyselleştirilmiş tedavi rejimlerini oluşturabilecek cazip adaylar sunmuştur.

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IIIA) Curriculum Vitae

Çiğdem Ercan was born on 1st of January 1982 in Eskişehir, Turkey, as the eldest of two children. In 2005, she received her bachelor diploma in Biology from the Middle East Technical University (METU) in Ankara, Turkey. After graduation, she started working at the Scientific and Technological Research Council of Turkey (TUBITAK) in Ankara, at the European Framework Programmes' National Coordination Office as a LifeScienceHealth Project Assistant. While she was working, she decided to expand her knowledge in Biology, in another country. In 2006, she was awarded with a study grant by the University of Groningen to do a research master in Medical Pharmaceutical Drug Innovation in Groningen, in the Netherlands. During her master education, she did two internships; in her first project, she investigated the role of RAC proteins in mediating the interaction between hematopoietic stem cells and bone marrow microenvironment at the Department of Internal Medicine, Division of Experimental Haematology at the University Medical Center Groningen (UMCG). During her second internship, she investigated the mutual regulation between lipid rafts and actin cytoskeleton in neuroblastoma cell lines at the Department of Cell Biology, Division Membrane Cell Biology at UMCG. In 2008, she graduated with a research master degree (cum laude) and soon after she started her PhD at the Department of Pathology of the University Medical Center Utrecht (UMCU) in the Breast Cancer Research Group of Prof. Paul van Diest. Her PhD project was focused on the association of several oncogenic pathways (NOTCH, p53, HIF-1α) and lobular breast cancer. The results obtained during her PhD are described in this thesis.

IIIB) Özgeçmiş

Çiğdem Ercan, iki kardeşin en büyüğü olarak, 1 Ocak 1982 tarihinde Eskişehir, Türkiye'de doğdu. 2005 yılında Orta Doğu Teknik Üniversitesi (ODTÜ), Biyoloji bölümünden şeref öğrencisi olarak mezun oldu. Mezun olduktan sonra, Türkiye Bilimsel ve Teknolojik Araştırma Kurumu'nda (TÜBİTAK), Avrupa Çerçeve Programları Ulusal Koordinasyon Ofisi'nde LifeScienceHealth Proje Asistanı olarak çalışmaya başladı. Çalışmaları sırasında, Biyoloji alanındaki bilgisini başka bir ülkede geliştirmeye karar verdi. 2006 yılında, Hollanda'da bulunan Groningen Üniversitesi tarafından "Medical Pharmaceutical Drug Innovation" adlı yüksek lisans programına katılmak için burs kazandı. Yüksek lisans öğrenimi esnasında Groningen Üniversitesi Akademik Hastahanesi'nde iki araştırma projesinde rol aldı; Deneysel Hematoloji Bölümü'nde yürüttüğü ilk projesinin konusu hematopoetik kök hücreleri ve kemik iliği mikroçevresi arasındaki etkileşimde RAC proteinlerinin rolüydü. Hücre Biyolojisi Bölümü'nde yürüttüğü ikinci projesi süresince nöroblastoma hücrelerinde lipid raft alanları ve hücre iskeleti arasındaki karşılıklı etkileşimi araştırdı. Yüksek lisans diplomasını 2008 yılında şeref derecesiyle aldı. Hemen ardından, Utrecht Üniversitesi Akademik Hastahanesi'nde, Prof. Paul van Diest'in meme kanseri araştırma grubunda doktora çalışmasına başladı. Doktora projesini lobüler meme kanseri ve bu kanserde rol oynayan onkojenik sinyal yolları üzerine yaptı, araştırması sırasında elde ettiği sonuçlar bu tezde yer almaktadır.

IV) Publication List / Yayınlar

2012

HIF-1α and NOTCH signaling in ductal and lobular carcinomas of the breast
Ercan C, Vermeulen J, Hoefnagel L, Bult P, van der Groep P, van der Wall E, van Diest PJ. Cell. Onco., *in press*

P53 Mutations in Classic and Pleomorphic Lobular Carcinoma of the Breast
Ercan C, van Diest PJ, van der Ende B, Hinrichs J, Bult P, Buerger H, van der Wall E, Derksen P. Cell. Onco., 2012 Apr;35(2):111-8.

Comprehensive Analyses of Kaiso Expression and Localization in Breast Cancer. Vermeulen J, van der Ven R, **Ercan C**, Bult P, van der Wall E, van Diest PJ, Derksen P. PloS One, 2012 7(5): e37864

2011

Mammary development and breast cancer: the role of stem cells.
Ercan C, van Diest PJ, Vooijs M. Curr Mol Med. 2011 Jun;11(4):270-85.

Multidrug resistance-related protein 1 (MRP1) function and localization depend on cortical actin.
Hummel I, Klappe K, **Ercan C**, Kok JW. Mol Pharmacol. 2011 Feb;79(2):229-40.