Aus dem Department diagnostische Labormedizin der Universitätsklinikum Tübingen

Abteilung Allgemeine und Molekulare Pathologie und Pathologische Anatomie

„Invasive Micropapillary Breast Carcinoma “

Inaugural-Dissertation
zur Erlangung des Doktorgrades
der Medizin

der Medizinischen Fakultät
der Eberhard Karls Universität
zu Tübingen

vorgelegt von
Warzecha (Geb. Nassar), Hind

2018

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Tag der Disputation: 10.07.2018

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Abbreviations

aPKC	atypical protein kinase C
AR	Androgen receptor
ASCO	American society of clinical oncology
ATS	American thoracic society
CAP	College of American pathologists
DCIS	Ductal carcinoma in situ
EGFR	Epidermal growth factor receptor
EMA	Epithelial membrane antigen
ER	Estrogen receptor
ERS	European respiratory society
HER2	Human epidermal growth factor receptor 2
IASLC	International association for the study of lung cancer
IHC	Immunohistochemistry
IMPCa	Invasive micropapillary carcinoma
ISH	In situ hybridization
LN	Lymph nodes
LVI	Lymphovascular invasion
MUC	Mucin
MRI	Magnetic resonance imaging
NST	No special type
T (stage)	Tumor (stage)
TMA	Tissue microarray
TN	Triple negative

TTF1	Thyroid transcription factor 1
WES	Whole exome sequencing
WGS	Whole genome sequencing
WHO	World health organisation

1. Introduction

1.1. Special subtypes of invasive breast carcinoma

1.1.1. Morphological classification of invasive breast carcinoma

Breast carcinoma has been traditionally classified into different subtypes based on its morphological features. The most common type of breast cancer, the previously called “invasive ductal carcinoma” is in fact not a particular tumor type. It has been re-named in the latest edition of the “WHO classification of the tumours of the breast” (2012) as invasive breast carcinoma of no special type (NST) emphasising its lack of particular morphological features and its extremely heterogeneous appearance (Lakhani et al. 2012). Invasive carcinoma NST constitutes approximately 75% to 80% of all invasive breast carcinomas [(Lakhani et al 2012), (Rosen 2009), (Tulinius et al. 1988)]. The remaining breast cancers belong to the so-called “special subtypes”. These constitute a long list with the most common subtype being invasive lobular carcinoma, and the rest including tumors such as invasive tubular carcinoma, metaplastic carcinoma, and invasive micropapillary carcinoma among others (Lakhani et al. 2012). A complete list of the special subtypes of breast carcinoma according to the WHO is included in List 1.

List 1: WHO classification of tumors of the breast (Lakhani et al. 2012)

Invasive carcinoma of no special type (NST)
- *Pleomorphic carcinoma*
- *Carcinoma with osteoclast-like stromal giant cells*
- *Carcinoma with choriocarcinomatous features*

Special subtypes
- *Invasive lobular carcinoma*
  - *Classic lobular carcinoma*
  - *Solid lobular carcinoma*
  - *Alveolar lobular carcinoma*
  - *Pleomorphic lobular carcinoma*
  - *Tubulolobular carcinoma*
  - *Mixed lobular carcinoma*
- *Tubular carcinoma*
- *Cribriform carcinoma*
- *Mucinous carcinoma*
- *Carcinoma with medullary features*
  - *Medullary carcinoma*
  - *Atypical medullary carcinoma*
  - *Invasive carcinoma NST with medullary features*

Carcinoma with apocrine differentiation
Carcinoma with signet ring cell differentiation
Invasive micropapillary carcinoma
Metaplastic carcinoma of no special type
Low-grade adenosquamous carcinoma
Fibromatosis-like metaplastic carcinoma
Squamous cell carcinoma
Spindle cell carcinoma
Metaplastic carcinoma with mesenchymal differentiation
Chondroid differentiation
Osseous differentiation
Other types of mesenchymal differentiation
Mixed metaplastic carcinoma
Myoepithelial carcinoma
Rare types
Carcinoma with neuroendocrine features
Neuroendocrine tumor, well differentiated
Neuroendocrine carcinoma, poorly differentiated (small cell carcinoma)
Carcinoma with neuroendocrine differentiation
Secretory carcinoma
Invasive papillary carcinoma
Acinic cell carcinoma
Mucoepidermoid carcinoma
Polymorphous carcinoma
Oncocytic carcinoma
Lipid-rich carcinoma
Glycogen-rich clear cell carcinoma
Sebaceous carcinoma
Salivary gland/skin adnexal type tumors
Cylindroma
Clear cell hydradenoma

1.1.2. Significance of the special subtype

Identification of special subtypes of breast carcinoma has been traditionally used as a tool to refine the prediction of behaviour and response to therapy in breast cancer provided by other morphologic prognostic parameters such as tumor grade, tumor size, lymph node status and lymphovascular invasion [(Carry et al. 2005), (De Mascarel 2008), (Ellis et al. 1992), (Jeruss et al. 2008), (Michaelson et al. 2003), Perry et al. 2008), (Rakha et al. 2008), (Tabar et al. 1996), (Vinh-Hung et al. 2003)]. Breast carcinoma subtypes have a characteristic morphology that typically correlates with a specific clinical behaviour. For example, colloid, tubular, and cribriform carcinomas, in their pure form (i.e. >90% of the tumor volume is composed of the special type) have a very good prognosis in comparison to invasive carcinoma NST

presenting at a similar pathologic stage [(Carstens et al. 1985), (Clayton 1986), (Fischer et al. 1980)]. Moreover, these types present usually at an early with negative or low count of positive lymph nodes (≤3) (Donegan 1997). Carcinomas with special morphology can be admixed with the invasive breast carcinoma NST. When the special pattern constitutes 50-90% of the tumor volume, the tumor is then termed mixed carcinoma NST and special type, for example “mixed invasive breast carcinoma NST and tubular carcinoma” (Lakhani et al. 2012). In this case the tumor loses some of its “special” behavioural characteristics as compared to the pure special subtype [(Norris et al 1965), (Page et al.1983)].

Other special morphologies do not confer to the tumor a different prognosis than that of invasive carcinoma NST -or at least the difference in prognosis is debatable-, but show a different type of presentation and behaviour. Invasive lobular carcinoma is a good example in this case [(Ashikari et al. 1973), (Cha et al. 2014), (Silverstein et al. 1994)]. It presents more often than other types as a bilateral or a multifocal tumor with a diffuse pattern of infiltration and therefore requires different pre-operative studies than invasive carcinoma NST to detect its extent [(Berg et al. 2004), (Chung et al. 1997), (Pestalozzi et al. 2008)]. In this case Magnetic Resonance Imaging (MRI) is more accurate than mammography and sonography and is used in the assessment of the preoperative extent of the disease and staging of the patients [(Kepple et al. 2005), (Mann et al. 2008)]. Moreover, the pattern of metastasis of invasive lobular carcinoma is different than that of breast carcinoma NST. The former has a higher affinity for visceral organs, bone, meninges, ovaries and serosal tissue [(Borst et al. 1993), (Dixon et al. 1991), (Harris et al. 1984), (Lamovec et al. 1991)].

In other cases the specific morphology when present indicates a worse clinical behaviour than that of invasive carcinoma NST. This is the case of invasive micropapillary carcinoma (IMPCa), which is the focus of this study.

1.2. Special subtypes vs. intrinsic subtypes of invasive breast carcinoma

1.2.1. Definition and classification of the intrinsic molecular subtypes

It is noteworthy that one of the most important prognostic factors that have emerged in the past few years is the molecular typing of breast cancer (Perou et al. 2000). Based on the molecular gene expression profiles and with the use of

immunohistochemical correlates, “intrinsic subtypes” of breast cancer have been identified [(Goldhirsch et al. 2013), (Goldhirsch et al. 2011), (Maisonneuve et al. 2014)]. These have important therapeutic consequences and prognostic implications (Goldhirsch et al. 2013). 1) Luminal A and 2) Luminal B subtypes are hormone receptor positive, 3) HER2 subtype is hormone receptor negative but HER2 positive, and 4) Triple negative subtype is hormone receptor and HER2 negative. Luminal B differs from Luminal A by a lower value of PR and/or a higher Ki67 proliferation index. Luminal B can be HER2 negative or positive (in addition to being hormone receptor positive). A detailed classification of the intrinsic subtypes according to the St-Gallen Consensus Conference 2013 is included in Table 1.

Table 1: Surrogate definition of intrinsic subtypes of breast cancer

Intrinsic subtype	***Clinicopathologic surrogate definition***
Luminal A	***Luminal A-like:*** *ER and PR+, HER2 –, Ki67 ‘low’*
Luminal B	***Luminal B-like HER2 negative:*** *ER +, HER2- and at least one of: Ki67 ‘high’, PR – or ‘low’,*
	Luminal B-like HER2 positive: *ER+, HER2+, any Ki67, any PR*
Erb-B2 overexpression	***HER2:*** *HER2 + and ER/PR -*
Basal-like	***Triple negative (ductal):*** *ER, PR and HER2-*

Notes: *1) The cut-point between ‘high’ and ‘low’ values for Ki-67 varies between laboratories. A level of <14% best correlated with the gene-expression definition of Luminal A based on the results in a single reference laboratory. Some of the participants preferred to use a cut off value of 20%. 2) A cut-point of <20% was used to characterise the “low” PR values.*

1.2.2. Significance of the intrinsic molecular subtypes

The classification of breast carcinoma into one of the four molecular subtypes is very important since each of these subtypes responds to a different targeted therapy [(Goldhirsch et al. 2013), (Goldhirsch et al. 2011)]. Endocrine therapy is used for the Luminal A subtype. The case of the Luminal B tumors is more complicated. These are treated with chemotherapy in addition to endocrine therapy and with HER2 targeted therapy when they are HER2 positive. Chemotherapy alone is usually administered in the case of triple negative breast cancer. For the HER2 positive subtype, chemotherapy is recommended in addition to HER2 targeted therapy (Goldhirsch et al. 2013).

1.2.3. Intrinsic molecular subtypes in relation to special subtypes

Although the molecular classification seems to have overshadowed the morphological classification and the traditional prognostic markers in breast cancer, the latter still have their importance. Moreover, there is a strong correlation between the special subtypes and the intrinsic molecular subtypes of breast cancer [(Caldarella et al. 2013), (Weigelt et al. 2008)]. For example, invasive lobular carcinoma belongs almost always to the Luminal subtype, so do tubular, cribriform and mucinous carcinomas [(Colleoni et al 2012), (Jung et al. 2010)]. Metaplastic carcinoma, carcinoma with medullary features and adenoid cystic carcinoma are overwhelmingly triple negative tumors (Reyes et al. 2013). However, despite their belonging to the same molecular type these tumors display different clinical behaviour and prognosis. Invasive lobular carcinoma has a worse behaviour compared to that of invasive tubular or invasive cribriform carcinoma. Adenoid cystic carcinoma of the breast, although triple negative, has an excellent prognosis with extremely rare metastatic potential, very different from that of the metaplastic carcinoma and carcinoma with medullary features [(Irshad et al 2011), (Montagna et al. 2013)]. In these cases tumor morphology conveys crucial information in addition to the molecular subtyping in regard to prognosis and therapy.

Therefore, despite the groundbreaking molecular advances in breast cancer, a careful study of the traditional characteristics of the tumor including morphology and the classification of breast cancer into one of the special subtypes or no special type (NST) remains necessary (Dieci et al. 2014). It remains also important to understand why a particular morphology confers to the tumor a particular behaviour.

1.3. Next generation sequencing (NGS) in the different types of invasive breast carcinoma

As cancer is a genetic disease driven by hereditary or somatic mutations, DNA sequencing is crucial in discovering these mutations. In recent years NGS technologies also called second- generation technologies have played a major role in understanding the altered genetic pathways involved in cancer. In comparison to earlier sequencing methodologies (Sanger sequencing), NGS is a high throughput method allowing in a relatively short time and at a relatively low cost massively to

parallel sequence millions of DNA templates. This can offer eventually a major advantage in terms of rapid diagnosis and the choice of targeted therapy when available.

Practically, there are multiple NGS platforms available, mainly from three companies (Roche, Illumina, and Life Technologies), differing by sequencing chemistry and methods for signal detection [(Chin et al 2011), (Meldrum et al. 2011)]. DNA sequencing includes whole-genome sequencing (WGS), whole-exome sequencing (WES), and gene-panel sequencing. Although mostly used for basic and clinical research, NGS is not only limited to this field. It is being increasingly useful in clinical practice mainly in solid tumors, where the detection of certain genetic mutations has a major impact on therapy.

A limited numbers of studies have been published to date on the application and utility of NGS in breast cancer. For an extensive overview we refer to the paper by Desmedt et al, which reviews the results of the largest four studies addressing this topic (Desmedt et al. 2012). In summary, these papers show that with the use of NGS new driver breast cancer genes are identified, those however being infrequent (<10% of the cases) compared to p53 and PIK3CA genes (>30% of the cases of ER negative and ER positive breast carcinomas respectively). On the other hand not all breast cancers harbour a driver mutation suggesting that other mechanisms such as DNA methylation can be involved in the pathogenesis of this disease. These studies also show that gene mutations are very heterogeneous in breast cancer but these however belong to a limited number of genetic pathways, a finding that can have major consequences on breast cancer targeted therapy. Lastly, they demonstrate that some mutations might be associated to the response or resistance to anticancer therapies.

In later studies, other researchers have shown that specific genetic alterations detected by NGS can be linked to specific breast cancer phenotypes (Russnes et al. 2011). For example breast cancer subtypes such as lobular and medullary carcinomas that are ER positive/HER2 negative and ER negative/HER2 negative respectively and belong to luminal A (lobular carcinoma) and basal-like (medullary carcinoma) molecular subtypes. In a further step recent NGS studies demonstrate that these two carcinoma subtypes also display different classes of mutations. To

date there is no data in the literature on NGS in invasive micropapillary breast carcinoma. The technique appears however to be very promising in explaining the morphology of this type of breast cancer and its behavior, which are very unique as it is detailed later.

1. 4. A special subtype of invasive breast carcinoma: Invasive micropapillary carcinoma

1.4.1. Historical perspective and terminology

The special subtype of IMPCa, as it is described in the next paragraph, have been first noticed in the 1980s by Fischer et al. who described this particular tumor configuration as “exfoliative” appearance and referred to these tumors as “pseudo papillary carcinoma” (Fischer et al. 1980). The name “Invasive micropapillary carcinoma” was first given in 1993 by Tavassoli et al. The authors described nine cases of breast carcinoma with this morphology and demonstrated the aggressive behaviour of the tumor (Siriaunkgul and Tavassoli 1993). In 1994 Luna-Moré et al proposed the recognition of IMPCa as a new entity (Luna-Moré et al. 1994). This term was officially recognised in 2003 in the 3rd edition of the WHO classification of tumors “Pathology and genetics of the Tumours of the Breast and the Female Genital Organs” (Tavassoli and Devilee 2003).

1.4.2. Description of the morphological features

The typical morphology of IMPCa consists of groups of breast cancer cells which are lacking, by definition, fibrovascular cores (as opposed to the term papillary), and are surrounded by clear spaces. Some authors believe these spaces are artefact of fixation (Tressera et al. 1999). In any case, although they resemble lymphatic channels they lack however endothelial cells as it was demonstrated by immunohistochemical staining [(Siriaunkgul and Tavassoli 1993), (De La Cruz et al. 2004), (Pettinato et al. 2004)]. The surrounding stroma shows no desmoplastic reaction but is typically described as “spongy” where thin strands of fibrous tissue separate the groups of tumor cells (Luna-More et al 1994). The mitotic rate and the cellular morphology are variable with moderate cellular atypia (Figure 1). Psammoma bodies have been described in almost half of the cases (42–62%) [(Middleton et al. 1999), (Pettinato et al. 2004)].

Defined as such, a pure invasive micropapillary growth pattern is rarely observed This pattern is usually mixed, in variable proportion, with invasive breast carcinoma NST or less commonly with other special subtypes of breast carcinoma [(Luna-More et al. 1994), (Walsh and Bleiweiss 2001)] (Figure 2). It has been also shown that most often, tumors with IMPCa retain the micropapillary growth pattern in lymphovascular spaces, as well as in lymph nodes and systemic metastases (Walsh and Bleiweiss 2001) (Figure 3).

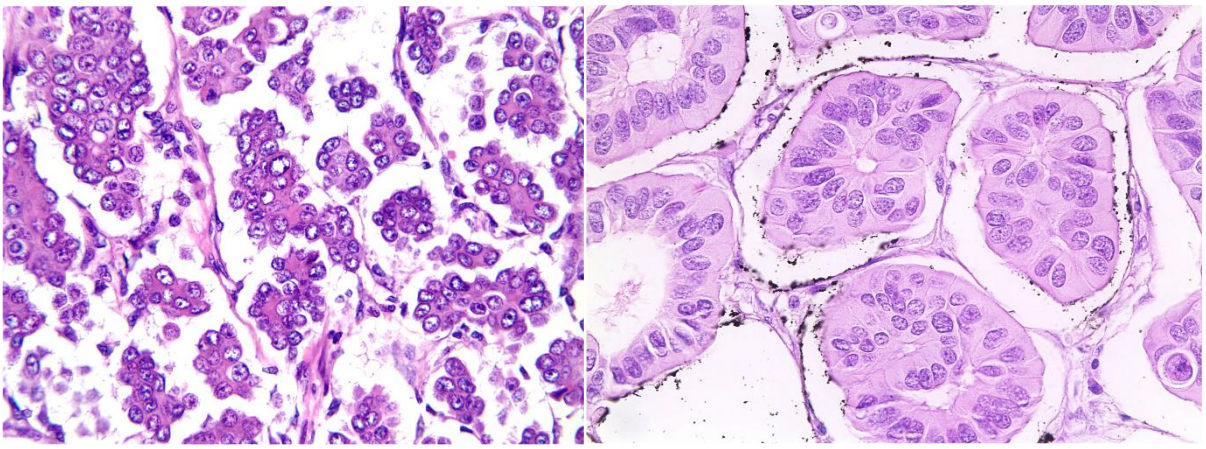

Figure 1: Invasive micropapillary carcinoma of the breast on a H&E section, low (left) and high magnifications (right)

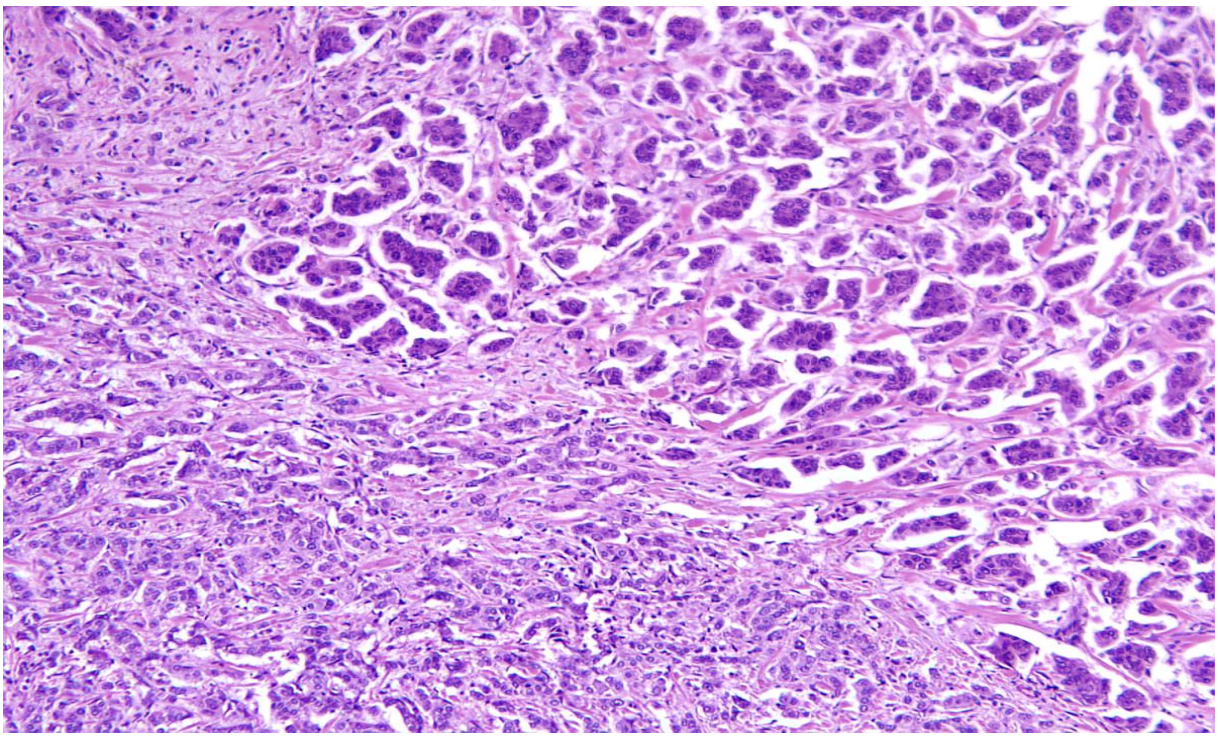

Figure 2: Invasive micropapillary carcinoma (right and upper part) of the breast admixed with invasive carcinoma no special type (left and lower part) at low magnification.

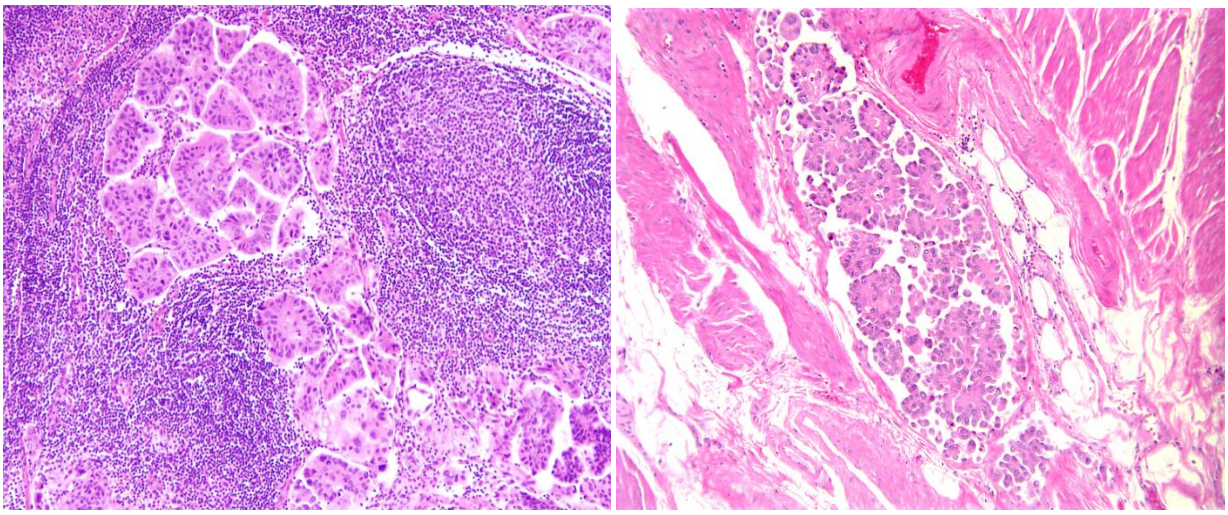

Figure 3: Invasive micropapillary carcinoma in metastatic sites; the invasive micropapillary morphology is preserved in lymph nodes (picture to the left) and in lymphovascular spaces (picture to the right), both at low magnification.

1. 5. IMPCa in organs other than breast

1.5.1. General information

Tumors with micropapillary morphology, although initially described in breast, occur in other organs such as the genitourinary tract, lung, gastrointestinal tract and salivary glands. In every organ system, these tumors have a marked propensity for lymphatic metastasis and an aggressive behaviour when compared to conventional carcinomas arising in these organs [(Amin and Epstein 2012), (Amin et al. 1994), (Amin et al. 2002), (Khayyata et al. 2005), (Sakamoto et al. 2005), (Shimoda et al. 2008)].

1.5.2. IMPCa of the genitourinary tract

This tumor occurs most commonly in the bladder where two distinct morphologic patterns of micropapillary carcinoma have been described: 1) an invasive pattern with the classic micropapillary morphology as described above and 2) a non-invasive component formed by slender filiform projections with or without thin fibrovascular cores resembling more the micropapillary serous carcinoma of the ovary [(Alvarado-Cabrero et al. 2004), (Amin and Epstein 2012), (Amin et al. 1994), (Johansson et al. 1999), (Loperz-Beltran et al. 2010), (Maranchie et al. 2000)]. In the bladder, IMPCa, same as in the breast, is almost always mixed with more conventional carcinoma,

papillary, invasive urothelial or adenocarcinoma and forms at least 20% of the tumor in most of the reported cases.

IMPCa of the bladder, even when present as a focal component is an aggressive tumor with a high frequency of lymphovascular invasion and muscle invasion at presentation (Compérat et al 2010). In general the outcome is poor [(Amin and Epstein 2012), (Watts and Hansel 2010)]. Therefore there is a tendency to treat this tumor with radical cystectomy even at an early stage (Willis et al. 2014). This has been however controversial [(Porten et al. 2014), (Wang and Wang 2013), (Willis et al. 2014)].

Few cases have been reported in the ureters with similar morphology and outcome as in the bladder (Radulović et al. 2012).

1.5.3. IMPCa of the lung

In the latest International Association for the Study of Lung Cancer (IASLC)/American Thoracic Society (ATS)/European Respiratory Society (ERS) classification system of lung tumors (2011), micropapillary predominant carcinoma is recognised as an independent subtype of adenocarcinoma (Travis et al. 2011). This inclusion was justified by the poor prognosis of this tumor documented in earlier reports even in resected stage I patients (Travis et al. 2011). In a recent study, Hung et al. show that micropapillary predominant adenocarcinoma had a higher recurrence rate with a higher frequency of developing extrathoracic-only recurrences in comparison with the other subtypes of lung adenocarcinoma (except for the solid predominant adenocarcinoma subtype which showed similar behaviour as invasive micropapillary carcinoma). In addition, the micropapillary subtype was a significant negative prognostic factor in overall survival, and disease specific survival (Hung et al. 2014). Similar findings were obtained by Cha et al. who showed that micropapillary (and solid) subtype is common in tumors greater than stage I. In their study, the presence of the micropapillary subtype was a single prognostic factor for overall survival (Cha et al. 2014).

Interestingly, IMPCa seems to grow as in the lung like in bladder in two different patterns which can be admixed: a common alveolar pattern where the tumor cells

float in alveolar spaces and a much less common stromal pattern where the IMPCa resembles that seen in breast (Ohe et al. 2012). On Univariate analysis both micropapillary patterns (alveolar and stromal) were significant predictors of unfavorable outcome. However, in multivariate analysis, among patients with stage I disease, patients with stromal but not alveolar micropapillary pattern showed a significantly poorer disease free survival than those without a micropapillary component (Ohe et al. 2012).

1.5.4. IMPCa of the gastrointestinal tract

IMPCa has been described in the colon, rectum, and stomach and less frequently in other locations such as the pancreatobiliary region. It is very rarely observed as a pure tumor or even as the predominant growth pattern in the tumor. Despite this observation, micropapillary morphology is associated in colorectal tumors with aggressive behaviour as demonstrated by a high frequency of lymphovascular invasion and lymph node metastases even in pT1 tumors. Stage I and II patients experience shorter survival as compared to the non-micropapillary groups (Guzińska-Ustymowicz et al. 2014). In many of these cases the micropapillary histology is pure or predominant in the metastases even though it does not constitute the predominant component in the primary tumor [(Kim et al. 2006), (Sakamoto et al. 2005), (Wen et al. 2008)]. In the colorectum 50% of the cases are diagnosed in the ascending colon whereas tumors in the rectum are limited to case reports.

This lesion is also rare in the stomach. In this organ, It has never been described as a pure tumor, however even a minor micropapillary component indicates tumor aggressiveness [(Eom et al. 2011), (Roh et al. 2010), (Ushiku et al. 2011)]. In the majority of cases, same as in the colon, there is lymphovascular invasion, lymph node metastases and an extension to the subserosa or to adjacent organs. Patients with IMPCa in the stomach have lower survival rates than those without a micropapillary component. It is still debatable whether or not the micropapillary morphology is an independent prognostic factor [(Ninomiya et al. 2013), (Roh et al. 2010)].

Khayyata et al found IMPCa in 4.1% of all cancers in ampullopancreatobiliary region, with the majority observed in the periampullary region (11% of all cancers in this

location), and the remaining in the pancreas (3% of pancreatic cancers) (Khayyata et al. 2005). Contrary to other locations, in the pancreas there was in association with the micropapillae an abundant inflammatory infiltrate composed mostly of neutrophils and less frequently of eosinophils that formed focal intraepithelial and stromal microabcesses (Khayyata et al. 2005). In most cases, local lymph node involvement was found [(Fujita et al. 2010), (Kitagawa et al. 2007)]. The same results regarding behaviour and patient outcome were observed in tumors arising in this location as in the colon and stomach, the number of cases is however very small (Khayyata et al. 2005).

1.5.5. IMPCa of the salivary glands

Rare cases of tumors with IMPCa component have also been reported in other organs such as salivary glands in association with salivary duct carcinoma [(Michal et al. 2000), (Nagao et al. 2004), (Yamamoto et al. 2008)]. They have been described in the parotid and the submandibular gland. In one series all patients had lymphovascular and perineural invasion as well as lymph node metastases and most patients died of disease shortly after diagnosis (Nagao et al. 2004).

1.6. Theories regarding the pathogenesis of IMPCa

1.6.1. The theory of "the inside out pattern" or reverse polarity

As we previously stated in our review article about IMPCa in the journal Advances in Anatomic Pathology (Nassar H. 2004): "It is believed that the morphology of IMPCa is due to a reverse of the polarity of the neoplastic cells where the stroma-facing surface (basal surface) of the cells acquires apical secretory properties (Luna-More et al. 1994). Peterson described this phenomenon as an inside-out growth pattern (Peterson 1993). This alteration in cell polarity was demonstrated by electron microscopic examination of a handful of cases showing the presence of a large number of microvilli at the surface of the neoplastic cells facing the stroma, a finding that usually characterises the luminal surface of benign glands (Luna-More et al. 1994)". At the molecular level we have demonstrated that proteins that are normally localised at the apical surface of glandular epithelial cells such as MUC1 are also abnormally expressed in IMPCa, a topic that we will detail next.

1.6.2. MUC1 glycoprotein and other molecules involved in polarity

Epithelial cells are highly polarized and the establishment and maintenance of this polarity is crucial to their function. The mechanisms that induce and are involved in maintaining this polarisation are very complex and controlled by multiple pathways. These pathways include a large number of molecules involved in protein to protein interactions. It is believed that proteins involved in apical polarity are different than those involved in basolateral polarity and that these play an antagonistic role in order for polarity to be maintained. In addition the orientation of the epithelial cellular polarity depends to high extent on the extracellular matrix (Yu et al. 2005). Studies of the apical domain have focused on two major complexes: the Crumbs complex and the PAR complex (Macara 2004). PARD3, PARD6, and atypical protein kinase C (aPKC) form a PAR complex that localizes to the apical TJs junctions and regulates apical junction formation. Other important molecules, a lipid phosphatase (PTEN) and a small GTPase (Cdc42) among others are also involved in this complex mechanism of epithelial polarisation. The end result of this polarisation is the localisation of specific molecules in specific cellular domains where they achieve their function. Among these are mucins or MUCs which are a heterogeneous group of highly glycosylated proteins constituting the major component of mucus [(Corfield 2001), (Gendler 2001)]. This family of proteins can be divided into transmembrane and secretory mucins. Transmembrane mucins are believed to regulate growth, adhesion, and motility of the cells [(De La Cruz et al. 2004), (Ringel and Lohr 2003), (Taylor-Papadimitriou et al. 2002)]. Among these is MUC1, or epithelial membrane antigen (EMA), expressed on the apical surface of normal glands in many tissues where it is believed to maintain lumen intergrity (Walker 1990).

MUC1 loses its normal apical location in cancer and localizes mostly to the cytoplasm as can be demonstrated by immunohistochemical studies. Aberrant MUC1 expression is associated with poor outcome [(Ceriani et al. 1992), (Hilkens et al. 1995), (Muir et al. 1991), (Ohuchi et al. 1995), (Rahn et al. 2001), (Walker 1990)]. In IMPCa, we described another aberrant expression of MUC1; it is consistently expressed on the stroma-facing surface of the cell, independent of the location of the tumor (Nassar et al. 2004). This finding could explain the characteristic morphology of the tumor. In fact, *in vitro* studies showed that a high expression of MUC1 could lead to a lower cell to cell adhesion as well as adhesion between cells and

extracellular matrix [(Hilkens et al. 1995), (Wesseling et al. 1996), (Wesseling et al. 1995)]. A higher MUC1 expression also results in contraction of collagen type I matrix and altered expression of epithelial cytokeratins (Hudson et al. 2001). Taking these findings into account in addition to our previous observations of the pattern of expression of MUC1 in IMPCa we hypothesised in a previous publication on the subject that this pattern of expression "may be responsible at least in part, for the detachment of the cells from the stroma, one of the main entity-defining features of IMPCa" (Nassar et al. 2004). This finding also confirms the impression that IMPCa" is characterized by an abnormality in cell polarity that occurs in a fashion that is not seen in conventional carcinoma. In fact, in IMPCa the surface of the tumor cells that faces the stroma acquires apical secretory properties" (Nassar et al. 2004). In practice, immunohistochemical staining for MUC1 is used to confirm the diagnosis of invasive micropapillary carcinoma (Figure 4).

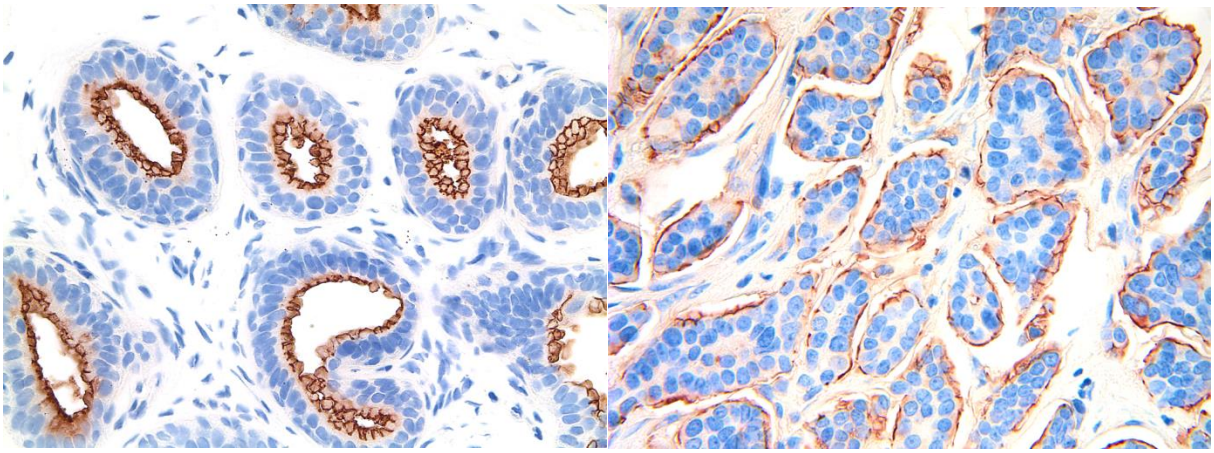

Figure 4: MUC1 immunostaining in benign breast terminal duct-lobular units (left picture) and in invasive micropapillary carcinoma (right picture) both at 20x magnification. In the benign terminal duct-units, the staining is at the luminal surface of the benign glands whereas in invasive micropapillary carcinoma MUC1 stains the stroma-facing surface of the malignant cell clusters (Taken from Nassar et al. 2004).

1.7. Aims of the study: extensive analysis of IMPCa at the clinical, immunohistochemical and molecular levels

1.7.1. Aim 1: description of the features of IMPCa of the breast at our institute

Our first aim is to provide a detailed description of IMPCa of the breast in our institute. The high yearly number (> 500) of newly diagnosed breast carcinomas at

the university hospital of Tuebingen allows us to get an overview of the frequency and characteristics of this relatively infrequent special type of breast cancer. More specifically we aim to characterise the morphology, expression of molecular markers, tumor behavior and outcome of the disease. There is to date no data on this type of breast cancer in the German population.

1.7.2. Aim 2: Ancillary immunohistochemical parameters in IMPCa and classification into molecular subtypes

Our second aim was to characterise IMPCa at the immunohistochemical level. These include studying 1) prognostic and predictive markers (hormone receptors, and Ki67-proliferation index, p53 and PTEN) and according to some of these markers classifying the IMPCa in one of the four molecular subtypes, 2) markers used to further characterise this type of tumor and help in its differential diagnosis from metastases (GATA3)

1.7.3. Aim 3: comparison of IMPCa to invasive carcinoma NST

It is important to know if this type of tumor has in our patient population significantly different pathological characteristic and behavior as that of invasive breast carcinoma, no special type.

1.7.4. Aim 4: molecular characteristics of IMPCa of the breast using next generation sequencing (NGS)

Our last aim was to check if these tumors harbor genetic alterations affecting known oncogens or tumor suppressor genes. Identification of these mutations may be able to explain the morphology and behavior of IMPCa and ultimately check the possibility for targeted therapy in this aggressive type of tumor. For this purpose we selected cases with pure IMPCa

1.8. Material and methods

Institutional Review Board approval was obtained from the Universitätsklinikum Tübingen

1.8.1. Patient cohort and clinicopathologic parameters

Breast cancer cases diagnosed at the Institute for Pathology at the University hospital of Tuebingen during the years 2003, 2004 and 2005 were reviewed. The study was limited to these three years in order to obtain a long-term follow-up. For the same reason i. e. the lack of reliable follow-up information, the cases referred from outside institutions (procedures that were performed outside the University Women's Hospital in Tuebingen) were excluded. We collected all breast cancer cases, first diagnosis and recurrences, diagnosed within this period of time. A total of 922 breast cancers were found.

For each breast cancer case we reviewed the available H&E sections and collected relevant data from the pathology reports, the patients' clinical electronic charts and cancer registry. After review of the sections we noted the morphological characteristics of the tumor including histological type (NST, special type or mixed), grade according to Ellston & Ellis, presence of lymphovascular invasion, presence of a DCIS component indicating if it is extensive, the latter being considered when DCIS constitutes more than 25% of the entire tumor mass. Tumor size, lymph node status, hormone receptors and HER2-oncoprotein status were collected from the pathology reports. All information regarding treatment (surgical procedure, endocrine, chemo, and radiation therapy) as well as follow-up data (local recurrence, distant metastases, and survival status) were obtained from the patients' charts and the cancer registry.

From this series, 43 cases of invasive carcinomas with predominant or focal micropapillary component were found. The specimens containing invasive micropapillary carcinoma consisted of resection specimens (excisional biopsies, simple mastectomies, modified radical mastectomies) and two needle core biopsies. Histologically, invasive micropapillary pattern was defined as first described by Siriaunkgul et al (Siriaunkgul and Tavassoli 1993). In addition to the parameters evaluated for all cases, we looked in these cases at the presence and type of the non-micropapillary invasive component. Lymphovascular invasion was considered to be present if tumor cells were found within a lymphatic space or a vessel in the stroma outside of the main tumor. Presence of micropapillary growth pattern was also assessed in axillary lymph node metastases.

1.8.2. Tissue microarrays and immunohistochemical staining

On a subset of cases (IMPCa and non- IMPCa) we performed tissue microarray (TMA) in order to repeat the immunhistochemical staining for hormone receptors and HER2- oncoprotein. Other immune markers were also performed on the same TMAs (MIB1, p53, GATA3, androgen receptor, and PTEN).

TMA was performed on randomly selected cases from the cohort. From each case we selected one or two tumor blocks and an additional block of non-involved breast tissue when the latter is not present on the tumor sections. TMA were manually constructed (BeecherInstruments MTA-1) from a total of 377 cases including 343 surgical resections and 34 core biopsies. Among these, 27 cases had micropapillary morphology. Surgical resection specimens were represented by six 1mm-diameter spots, four from neoplastic and two from non-neoplastic breast tissue. Core biopsy cases were represented by 2-4 spots of tumor depending on the tumor amount.

Immunohistochemical staining was performed using BenchMark XT IHC/ISH autostainer (Ventana Medical Systems, Roche; Tucson, Arizona). Briefly, 2.5μm sections were deparaffinised and subjected to heat-induced antigen retrieval using EDTA buffer (pH 8.4) for 64 minutes (standard CC1).Subsequently, incubation with the primary antibody (in the 37° temperature for 32 min) was followed by detection of reaction using iVIEW DAB (diaminobenzidine hydrochloride) v3 kit. All slides were counterstained with hematoxylin (4 min incubation). The sources, conditions and dilutions of the various antibodies used are as follows: ER (clone SP1 form Roche, rabbit monoclonal, ready to use), PR (1E2, form Roche, rabbit monoclonal, Ready to use), androgen receptor (AR441, form Dako, mouse monoclonal, dilution 1:200), HER2 (clone 4B5, from Roche, rabbit monoclonal, ready to use), Ki67 (clone MIB1, form Dako, mouse monoclonal, dilution 1:200), p53 (clone DO-7 form Novocastra, mouse monoclonal, dilution 1:200), GATA3 (clone L50-823 form Biocare, mouse monoclonal, dilution 1:500), and PTEN (clone 138G6, from Cell Signaling, rabbit monoclonal, 1:50). Fluorescent in situ hybridization (FISH HER2-Gen; Chromosom17 PathVysion-Kit, Abbott-Molecular) was performed in cases where HER2 immunostain was equivocal (see below). For ER, PR, androgen receptor (AR), and GATA3 we noted semi-quantitatively the percentage of cells with nuclear staining and the intensity of staining (weak 1+, moderate 2+, strong 3+). The percentage of nuclear positivity was noted for MIB1 and p53. HER2 was scored as positive (3+), negative (0

or 1+) or equivocal (2+) according to the new ASCO/CAP criteria using IHC and FISH (when indicated i.e. in IHC 2+ cases) (reference). PTEN was score as negative (0 or 1+ staining) or positive (2 or 3+ staining). The technical aspects of the immunohistochemical stains are summarised in Table 2. According to the immunhistochemical stains (ER, PR, HER2-Oncoprotein, and MIB1) we classified the tumors in one of the four molecular types (Luminal A, luminal B, HER2 and TN) according to the criteria described in the introduction.

Table 2: Characteristics of the Antibodies used in the study

	Clone	***Manufacturer***	***Dilution***	***Staining pattern***
ER	*SP1*	*Roche*	*Ready to use*	*Nuclear*
PR	*1E2*	*Roche*	*Ready to use*	*Nuclear*
AR	*AR441*	*DAKO*	*1:200*	*Nuclear*
HER2	*4B5*	*Roche*	*Ready to use*	*Membranous*
Ki67	*MIB1*	*DAKO*	*1:200*	*Nuclear*
P53	*DO-7*	*Novocastra*	*1:200*	*Nuclear*
GATA3	*L50-823*	*Biocare*	*1:500*	*Nuclear*
PTEN	*138G6*	*Cell Signaling*	*1:50*	*Cytoplasmic and nuclear*

1.8.3. Molecular studies using NGS

Genomic DNA was extracted from 5 µm paraffin sections using the Maxwell® 16 FFPE Tissue LEV DNA Purification Kit and the Maxwell® 16 Instrument (Promega, Madison, WI, USA) according to the manufacturer's instructions. Isolated DNA was quantified using the Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Targeted multigen mutation screening was performed by Next Generations Sequencing (Ion Torrent PGM, Thermo Fisher Scientific) using the AmpliSeq Cancer Hotspot Panel v2 (hotspot regions in 50 genes: ABL1, EZH2, JAK3, PTEN, AKT1, FBXW7, IDH2, PTPN11, ALK, FGFR1, KDR, RB1, APC, FGFR2, KIT, RET, ATM, FGFR3, KRAS, SMAD4, BRAF, FLT3, MET, SMARCB1, CDH1, GNA11, MLH1, SMO, CDKN2A, GNAS, MPL, SRC, CSF1R, GNAQ, NOTCH1, STK11, CTNNB1, HNF1A, NPM1, TP53, EGFR, HRAS, NRAS, VHL, ERBB2, IDH1, PDGFRA, ERBB4, JAK2, PIK3CA).

In a primary step, the Ion AmpliSeq Library Kit v2.0 was applied to amplify hotspot target regions specified by the panel. Subsequently primer sequences were partially

digested using the FuPA reagent. Each sample was marked by barcodes ligating Ion Xpress™ Barcode Adapters which include sequencing adapters. Libraries were purified and quantified applying Agencourt® AMPure XP (Beckman Coulter, Brea, CA, USA) magnetic beads and the Ion Library Quantitation Kit (Thermo Fisher Scientific) on the LightCycler 480 real-timer PCR system (Roche Molecular Systems, Pleasanton, CA, USA). Libraries were diluted to 100 pM each and pooled. In the next step DNA fragments were attached to Ion Sphere Particles (ISPs) and clonally amplified using the Ion PGM™ Hi-Q™ OT2 Kit and the Ion OneTouch™ Instrument. The amount of template-positive ISPs was determined with the Qubit® 2.0 Fluorometer and the Ion Sphere™ Quality Control Kit. Afterwards the Ion OneTouch™ ES was used to enrich template-positive ISPs. In a last step, sequencing primers were attached to the DNA fragments bound to the ISPs which were subsequently loaded on semiconductor chip (Ion 318™ Chip Kit). Finally sequencing was performed suing the the Ion PGM™ Hi-Q™ Sequencing Kit and the Ion Torrent PGM™ platform.

Detection of non-synonymous variants compared to the human reference sequence (hg19) was performed using the Torrent Suite™ and the Ion Torrent Variant Caller. Detection thresholds were set at an allele frequency of 5%. Variants were annotated and filtered against the dbSNP and COSMIC databases using the Annotate variants single sample workflow of the Ion Reporter Software (Thermo Fisher Scientific). Obtained variants were further visualized with the freely available program Integrative Genomics Viewer (IGV, Broad Institute) to discriminate artefacts and true variants.

1.8.4. Statistical analysis

A comparison between IMPCa and IMPCa invasive carcinomas was performed. We compared the following clinicopathologic parameters: T stage, LN status, recurrence/metastases, and survival status. Since the majority of IMPC tumors were ER positive we limited the comparison to these cases with ER positive non-IMPC cases (n=186). Statistical analyses were performed using JMP 12.2.0 (SAS Institute, Cary, NC, USA). Categorical variables were compared with Pearson's chi-square correlation calculated on contingency tables. Continuous variables were analyzed

using the Wilcoxon rank sum and chi-square test. A p value less than 0.05 was considered statistically significant.

2. Results

2.1. Description of the clinicopathological parameters of IMPCa of the breast:

Cases of breast carcinoma with pure or partial micropapillary differentiation formed 4.7% (43 patients) of the entire cohort of breast carcinomas diagnosed between 2003 and 2005 at our institute. The women were aged between 31 and 87 years with a median age of 63 years. The majority of the patients were postmenopausal (90%).

Histologically, in nine cases the tumor had pure micropapillary morphology (21%). In the other 34 cases, components of invasive carcinoma no special type (NST), lobular carcinoma or mucinous carcinoma were present; components from other special types of breast cancer were not observed. In two cases the NST component displayed a nested pattern reminiscent of neuroendocrine tumors. In a subset of cases (n=4; 9%) the tumor had at the same time mucinous and micropapillary morphology. Since according to literature (see introduction) those behave more like IMPCa than mucinous carcinoma, they were included in the IMPCa group of tumors. In these cases the micropapillae appear to be floating in mucin rather than being in empty spaces. Lymphovascular invasion (LVI) as defined in the material and methods was seen in 6 cases (14%). In regard to the Elston & Ellis tumor grade, almost all cases were G2; two cases displayed a G3 and one case was G1. A ductal carcinoma in situ (DCIS) component was seen in a quarter of the cases (n=11), being regarded as extensive in two of them. DCIS was typically intermediate grade with predominant micropapillary morphology. Of the 43 cases, 38 were first diagnosis tumors; in five cases (11.6%) the tumor represented a local recurrence of a primary carcinoma that was diagnosed between 1989 and 1996. For these five cases, the slides of the original tumors were not available for review and the original histology was unknown in the patients' clinical history.

The size of the tumors was available in 40 cases and varied between 0.5 to 4.5 cm with a mean size of 1.9 cm and a median of 2.0 cm. Pathologic T stage was

distributed as follows: 2.5% pT1a (n=1), 15% pT1b (n=6), 35% pT1c (n=14), 42.5% pT2 (n=17), 2.5% pT3 (n=1) and 2.5% pT4 (n=1). Multifocality/multicentricity was seen in 30% of the cases (n=12). These multifocal carcinomas belonged mostly to the pT2 stage followed by pT1c with one case staged as pT3. In regard to staging of the axilla, more than half of the patients (55%) had lymph nodes metastases. When the axillary lymph nodes were involved the number of positive nodes was more often greater than three. The distribution of axillary lymph nodes staging was as follows: 2 cases pN1mic, 8 cases pN1a, 8 cases pN2a, and 3 cases pN3a. Less than half (44.5%) were node negative; there was no lymph node status available in nine cases. In the involved lymph nodes, the metastases exhibited micropapillary morphology at least focally.

Information about treatment obtained from the patients' charts and the Tuebingen cancer registry showed that 20 patients were treated with partial breast resection and 20 had mastectomy either as initial procedure or after positive margins on an excisional biopsy. In addition, 38 patients had lymphadenectomy (88%). Regarding additional therapy, 27 patients received hormonal treatment, 26 patients underwent radiation therapy and 14 patients underwent chemotherapy. In other terms and for reasons that we were not able to identify 66% of the patients received endocrine therapy despite that 86% of them had positive hormone receptors. As far as combination of therapies, 12 patients were treated with radiation and endocrine therapy, seven patients had a combination of all three treatment modalities, five received endocrine therapy alone, 4 patients were radiated and had chemotherapy, 3 patients were treated with radiation alone and 3 other patients received chemotherapy in addition to endocrine therapy. Seven of the patients did not receive any treatment besides surgery.

Follow-up data was available in all but two patients (95%). Fifteen patients (15/41; 36.5%) developed distant metastases either at presentation (2 patients) or after few months to 11 years of the breast cancer diagnosis. Four of these patients did not have any positive lymph nodes; one had a micrometases in one lymph node. In five cases there was no information on lymph nodes. Eleven of the patients died from breast cancer between three to 10 years after the initial diagnosis.

2.2. Immunohistochemical staining

2.2.1. Hormone receptors and HER2 oncoprotein

Hormone receptors and HER2-oncoprotein status was available from the pathology reports for all patients. Thirty seven tumors were hormone receptor positive (86%). Among these three were also positive for HER2- oncoprotein. Four cancers were positive for HE2- oncoprotein and negative for hormone receptors (9.4%), two of them with a score of 2+ by immunohistochemistry and a detectable HER2 gene amplification by FISH. Two tumors were negative for all three markers (4.6%). Of note all the invasive carcinomas with pure micropapillary morphology were ER and PR positive without expression of HER2 oncoprotein.

Immunostaining for ER, PR and HER2- oncoprotein was repeated on the TMA in 27 cases of IMPCa. In general, there was no change of the hormone receptors status in any of the cases. For ER and HER2 the results did not differ from those retrieved from the pathology reports. For PR, the value was lower in five of the TMA cases in comparison to that seen in the reports staying within the positive range however (change from strongly positive in the reports to weakly positive in the TMA).

Androgen receptor (AR) was negative in half of the cases that we were able to evaluate (13/26). The staining was weakly positive (in 5-10% of the tumor cells) in 27% of the tumors (7 cases). The rest showed a positive labeling in >10% of the tumor cells, this labeling being diffuse only in a minority of cases (3 cases, 11%). All except two of the AR negative cases were ER positive, six of them with strong ER positivity. On the other hand the one IMPCa case that was ER negative (HER2 positive) on TMA was also AR negative.

2.2.2. Proliferation, p53 expression and PTEN

The IMPCa expressed a low Ki-67 proliferation index (lower than 10%) in the vast majority of cases (89%). In three cases the value was greater than 15%, being 15%, 20% and 30% in one case each. P53 was not overexpressed in 85% of the cases.

Two cases of luminal B type and two cases of TN molecular type showed an expression of p53 of 20-50%. The expression of PTEN was lost in 42% of IMPCa.

2.2.3. Expression of GATA3

GATA3 was positive in 20 of 23 stained cases (87%) with a moderate to strong intensity and with a percentage of positive cells that varies from 30 to 100%. Of the cases that were negative for GATA3, two cases were also triple negative and one was negative for ER and PR but positive for HER2.

2.2.4. Molecular classification

With the use of the surrogate immunohistochemical staining (ER, PR, HER2 oncoprotein, MIB1) we divided the cases into the four molecular subtypes using the recommendations of the saint Gallen consensus conference already mentioned in the Introduction. In the cases where no MIB1 staining was available we classified the cases based on the values of ER, PR and HER2 only. In summary, 55.6% (n=23) of the cases were luminal A, 21% (n=9) were luminal B-HER2 negative, 9.4% (n=4) were HER2 positive-luminal B, 9.4% (n=4) were HER2 type and 4.6% (n=2) were triple negative (TN) type.

2.3. Molecular characteristics of selected pure IMPCa

Using our 50 genes panel on six IMPCa cases with a pure invasive micropapillary component showed that three tumors were wild type for the genes tested and three others showed hotspot mutations.
In one case we detected a missense mutation of the PIK3CA gene which affected a known Hotspot (c.3140 A>G) corresponding to the amino acid change p.H1047R. The allele frequency was 18%.
In another case PIK3CA was wild type. A missense mutation of the TP53b gene (nucleotide change, c.637C>T and amino acid change p.R213) in addition to a mutation of the MET gene (nucleotide change, c.3029C>T and amino acid change p.T1010I) with a frequency of 33% and 35% respectively.

In the third case of the non-wild type IMPC a mutation of ABL1 gene was detected with a frequency of 48% affecting the c.797A>G and the amino acid p.K266R. The latter is believed to be a germline mutation.

2.4. Comparison of IMPCa with invasive breast carcinoma NST

As mentioned in the material and methods we compared the cases of IMPCa to the cases of invasive breast carcinomas NST in regard to staging (pT stage and lymph node status), risk of recurrence and distant metastases, survival, and the expression of specific immune markers. Since the majority of the IMPCa cases expressed ER, we limited the comparison to the ER positive tumors in both the IMPCa and the NST group. In other terms, at the molecular level the comparison was done between the luminal A and B cases in the IMPCa and the NST groups. The latter consisted of 219 breast cancers.

When comparing the stage at presentation, in our cohort, IMPC tumors had a statistically comparable pT stage distribution to that of NST breast cancers (pT 1mic +pT1a+ pT1b: 24%; pT1c: 41%, pT2: 31% and pT3 2% and pT4: 2%). Lymph nodes involvement however was overall significantly higher in IMPCa cases (p 0.02).

The risks for distant metastases and /or local recurrence as well as the risk of death of disease were clearly higher in IMPC than in NST breast cancer cases. This occurred in 22% of the breast cancers with IMPCa whereas 11% of NST breast cancers recurred or displayed distant metastases ($p < 0.0001$). This difference is also statistically significant when matching for node status (p 0.005).

We compared the distribution of the molecular subtypes (luminal A, luminal B-HER2 negative, and luminal B-HER2 positive) in NST and IMPCa breast carcinomas. In both groups luminal A constituted the majority of the (ER positive) cases with 65% in IMPCa and 62% in NST. This was followed by luminal B-Her2 negative with 25 %and 28% respectively. Luminal B-HER2 positive cases formed 10% of each category. This distribution did not show a statistically significant difference between the two categories.

At the immunohistochemical level, the expressions of p53 as well as the Ki67 proliferation index were similar between the IMPCa and NST breast cancer cases. The expression of PTEN was lost in 42% of IMPCa and 37% of NST tumors, showing no statistically significant difference.

3. Discussion

3.1. Summary of the results

The results of our study show that: 1) Invasive micropapillary morphology, which is seen in 90% of the cases in postmenopausal women, is not a very rare finding in breast cancer (approximately 5% overall). However, when present, it usually constitutes less than half of the tumor volume; pure invasive micropapillary carcinoma is seen only in one fifth of the cases. Regardless of extent, micropapillary differentiation is associated with a high rate of lymph nodes metastases and an aggressive behavior compared with invasive breast carcinoma NST. This confirms previous reports on this type of breast cancer in other patient populations. 2) The vast majority of the tumors belong to the luminal subtype with a high proportion of luminal B tumors. This distribution however was not different from that of the invasive carcinoma NST in our cohort. In addition and contrary to the findings in the literature Ki67 proliferation index was not higher in IMPCa. 3) A third of the pure IMPCa showed genetic alterations affecting TP53 or PIK3CA.

3.2. Morphology of IMPCa of the breast and differential diagnosis

3.2. 1. Morphological features

As previously mentioned the majority of invasive micropapillary carcinomas in our series were admixed with invasive carcinoma NST. Less often however they were described in other reports to be admixed with other special subtypes of invasive breast cancer including tubular, papillary, mucinous or invasive lobular carcinoma [(Lopez-Beltran et al. 2010), (Pettinato et al. 2004), (Walsh and Bleiweiss 2001)]. When diagnosing a case of breast cancer where micropapillary features are noted, it is important to mention in the diagnosis the presence of any micropapillary component since even a minimal one can have negative prognostic implications. Ductal carcinoma in situ (DCIS) has been reported in 53% to 80% of the cases being

described as extensive in half of them [(Luna-More et al. 1994), (Middleton et al. 1999), (Tressera et al. 1999), (Walsh and Bleiweiss 2001)]. DCIS is usually intermediate to high grade, often of micropapillary type but also cribriform or papillary types, with or without central necrosis (Figure 5). The identification of DCIS in a setting of IMPCa of the breast favours a primary carcinoma arising in this organ over metastases to the breast from IMPCa arising in other localisations, which can look identical.

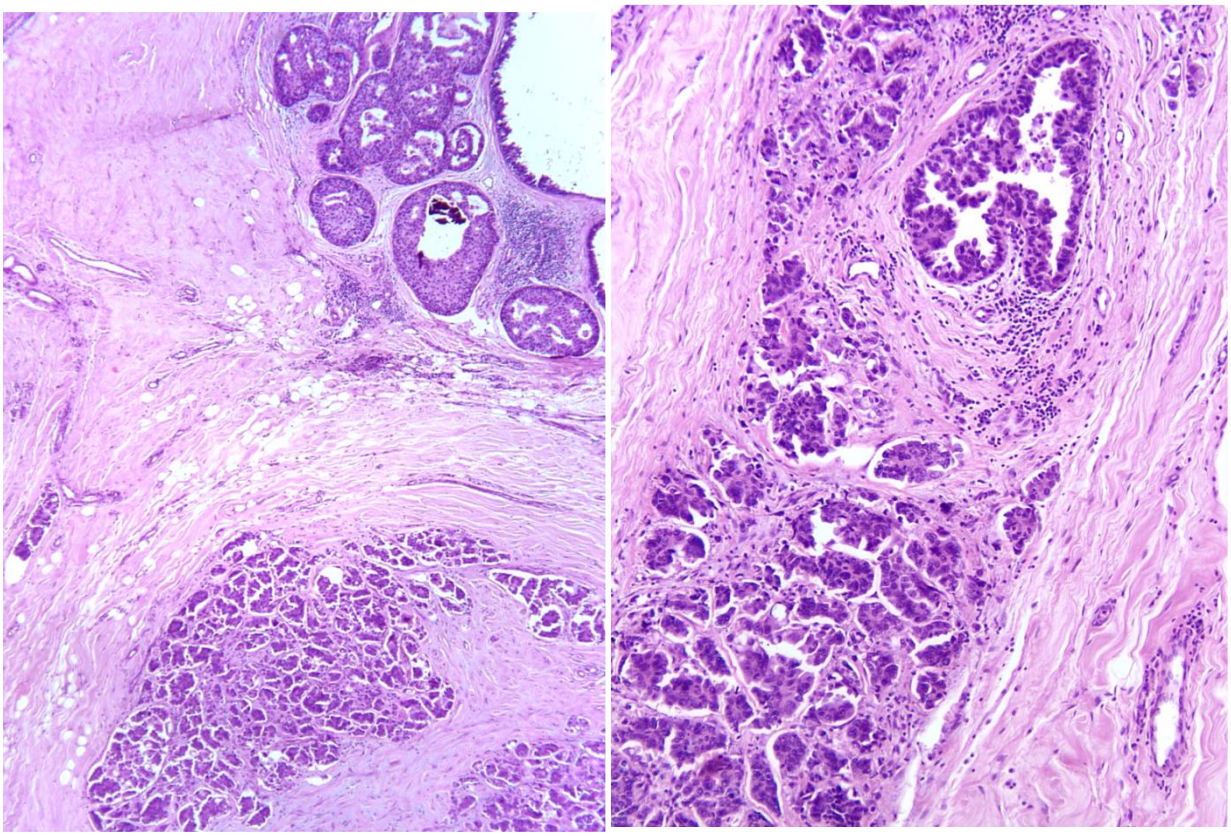

Figure 5: IMPCa of the breast in association with DCIS displaying micropapillary (picture to the left), cribriform and solid patterns with focal necrosis and *microcalcifications* (picture to the right), both at low magnification.

3.2.2. Differential diagnosis

3.2.2.1 Papillary carcinoma

The term “papillary” describes an epithelial proliferation with fibrovascular cores where the apical surface of the neoplastic cells faces opposite to these cores. The term “micropapillary” is defined by a proliferation of neoplastic cells also with

reversed polarity of the secretory poles but without fibrovascular cores. Therefore IMPCa of the breast is not related to the special type of encapsulated papillary carcinoma of the breast. “The latter typically exhibits a complex arborescent growth pattern within cystically dilated spaces” (Fischer et al. 1980). This morphology is very different from the typical morphology of IMPCa.

3.2.2.2. Mucinous carcinoma

Mucinous carcinoma with micropapillary pattern has been described as a rare type of tumor exhibiting simultaneously mucinous and micropapillary features. Barbaschina et al emphasise the importance of recognising this tumor type in a paper where they described 15 cases of mucinous breast carcinoma (>90% mucinous pattern) with a micropapillary arrangement of the neoplastic cells. They showed that morphologically these tumors had intermediate to high nuclear grade, hobnail cells and frequent psammoma bodies. They were characterised by an aggressive behaviour relative to that of the usual type of mucinous carcinoma translated by a high frequency of lymphovascular invasion and lymph node metastases (Barbaschina et al. 2013). In the current series we came across four cases of invasive micropapillary mucinous carcinoma (9%) and included them in the IMPCa category (Figure 6).

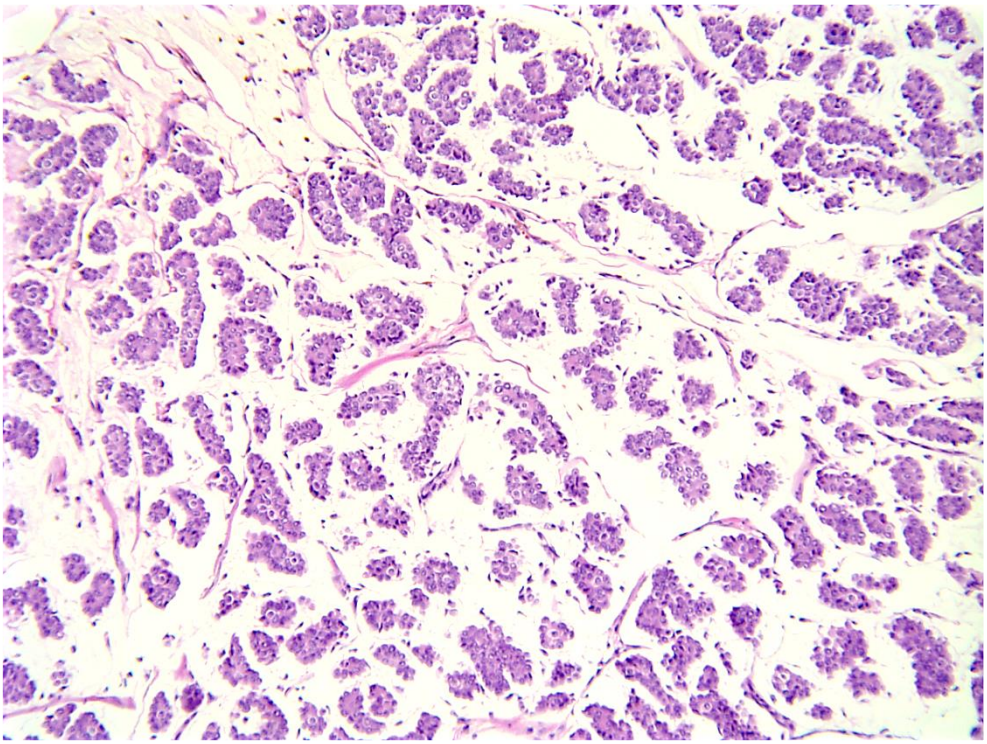

Figure 6: Mucinous carcinoma of the breast with micropapillary pattern

3.2.2.3. Retraction artefact

Previous reports draw attention to the so-called retraction artefact in invasive breast cancer and describe it as occurring in association with a dense collagenous stroma and therefore should be differentiated from the clear spaces within the loose stroma of IMPCa. Retraction artefacts were assumed to be due to tissue fixation. In recent publications, Acs et al demonstrated that these are in fact not “artefacts “ of fixation but a real phenomenon that happens with certain breast cancers since they can be seen not only in resection specimens but also on frozen sections and on needle core biopsies [(Acs et al. 2007), (Acs et al. 2009)]. More importantly they reported that tumors with these areas of retraction have a larger size, a higher grade and a high frequency of lymph node metastases (Acs et al. 2012). Their findings were validated in a large prospective series of breast cancer cases where the extent of retraction correlated significantly with the above mentioned parameters as well as nodal metastases (Acs et al. 2014). Their extensive presence in a tumor predicted poor recurrence free survival and overall survival in node negative and node positive cases. They concluded therefore that retraction artefacts are a morphological translation of a biological change leading to frequent lymphovascular invasion and lymph node metastases even at an early stage. We assume from the description of their cases and the figures in the paper that IMPCa is included in this category i.e. the category of “breast carcinomas with extensive retraction clefts”, an opinion that might not be shared by other authors.

3.2.2.4. Metastatic IMPCa to the breast from other organs

Rarely metastases from other anatomic locations can occur in the breast. When these metastases originate from a tumor with a micropapillary component or a pure IMPCa they can manifest as IMPCa of the breast and the differential diagnosis between a metastases and a primary IMPCa of the breast can be difficult. Particularly challenging are metastases to the breast (or the axillary lymph nodes) from serous carcinomas of the peritoneum, ovary or fallopian tube, a possibility that should be kept in mind. In addition to clinical information, the morphological features of the tumor such as presence of DCIS and in some cases immunohistochemical stains can be helpful in this setting. Recine et al showed that the use of immunohistochemistry can help in the differential diagnosis; WT1 is usually

expressed in serous carcinomas arising in these organs but not in a breast primary (Recine et al. 2004). Lotan et al found that the best immunhistochemical panel to determine the primary site of IMPCa includes Uroplakin, CK20, TTF1, ER, WT1 and/or PAX8, and mammaglobin (Lotan et al. 2009). IMPCa of lung is usually positive for TTF1 (and CK7) and negative for CK20 [(Amin et al. 2002), (Lotan et al. 2009)]. When the tumor is positive for Uroplakin and CK20 an urothelial origin can be favored [(Amin et al. 1994), (Lotan et al. 2009)]. Breast IMPCa is ER positive, mammaglobin positive, and PAX8/WT-1 negative, while ovarian carcinoma is ER positive, mammaglobin negative, and PAX8/WT-1 positive (Lotan et al. 2009). These studies were performed before the relatively recent widespread use of GATA3 as marker of breast origin. We found it to be positive in most of our cases and should be added to the above mentioned panels in the differential diagnosis. It is noteworthy that GATA3 is also a marker of urothelial differentiation and hence is not helpful in differentiating an IMPCa of the breast from that of urothelial origin.

3.3. Prognostic and predictive molecular features

The majority of IMPCa are ER and PR positive with varying proportions according to different studies, reaching 100% in some studies [(Luna-More et al 1996), (Machió et al. 2009), (Marchió et al. 2008), (Paterakos et al. 1999), (Tressera et al. 1999), (Walsh and Bleiweiss 2001), (Yamaguchi et al. 2008)]. In our study the 86% of the cases were hormone receptor positive. A positive-HER2 expression has been demonstrated in 10 to 35% of cases. It is important to note that the ASCO/CAP recommends classifying as weakly positive or equivocal by immunohistochemisty (2+) carcinomas with circumferential intense and complete membranous staining in < 10% of the tumor cells. IMPCa is an exception to this rule; in this tumor type a cup-shaped intense staining and not a complete membranous staining is often seen and should be counted as positive (Wolff et al. 2014). Due to its hormone receptor positivity, IMPCa belongs in most cases to the luminal subtype of breast cancer. In addition it is characterized by a high Ki67 labeling index. According to Vingiani et al, in approximately 90% of these tumors the Ki67 labeling index is >14% and in two third of the cases >20% and therefore classified as luminal B (Vingiani et al. 2013). Marchio et al. demonstrated that the genomic changes in their series of IMPCa cases were similar to those described in the luminal B subtype. This was true for pure IMPCa as well as those admixed with invasive carcinoma NST [(Marchió et al. 2009),

(Marchió et al. 2008)]. The expression of basal markers (CK5/6, CK14, CK17, EGFR) in this tumor is extremely rare [(Kim et al. 2006), (Marchió et al. 2008), (Marchió et al. 2009), (Weigelt et al. 2008)]. Our results are different than those reported and show that most IMPCa display a low proliferation index. Moreover the proportion of the luminal B cases, although relatively higher than that described for NST in the literature, was not different than that of the invasive carcinoma NST group in our series. The explanation could be that MIB1 immunostaining was performed on TMAs and therefore the tissue was not representative of the entire tumor. This is a problem with MIB1 since the hot spot areas should be reported. Our data show that p53 was not overexpressed in most of the cases. Like MIB1, it was high in the TN case.

3.4 Clinical and radiological presentation

At the clinical level, similar to invasive carcinoma NST, the most common manifestation of IMPCa is a palpable mass (60-94%), the rest being detected at screening mammography [(Adrada et al. 2009), (Günhan-Bilgen et al. 2002)].

Not many studies are available that extensively describe the radiological appearance of IMPCa of the breast. From these studies it appears that the radiological findings of IMPCa of the breast are not very specific. On mammography, these tumors typically appear as a high-density mass with spiculated margins that is often associated with microcalcifications. Microcalcifications with and without an associated mass or focal asymmetry were found in two thirds of the tumors in the study by Adrada et al. and in 43% in the study by Günhan-Bilgen et al [(Adrada et al. 2009), (Günhan-Bilgen et al. 2002)]. These microcalcifications are most likely associated with the accompanying DCIS component. Ultrasound studies usually reveal an irregularly shaped solid hypoechoic mass with indistinct margins without posterior acoustic shadowing or enhancement. Sonography is important in identifying lymph node metastases, it was able to identify approximately three quarters of axillary lymph nodes metastases in the same study (Adrada et al. 2009). MRI appearance of IMPCa was described in few cases and is considered important for defining the extent of the disease, multifocality, and tumor in the contralateral breast and therefore can play same as in invasive lobular carcinoma an important role in staging of the patient and subsequent disease management [(Adrada et al. 2009), (Lim et al. 2013)].

3.5. Clinical behavior and survival

Almost all studies describing IMPCa of the breast point that this tumor is characterized by a high degree of axillary lymph node involvement. For example, Luna-More et al. and Paterakos et al. reported 91% and 95% incidence of axillary node metastases respectively [(Luna-More et al. 1994), (Paterakos et al. 1999)]. When positive multiple lymph nodes are typically involved. We have shown in one series of IMPCa cases from a patient population treated in a tertiary health care centre in the USA that an average of six positive axillary nodes was detected. This number was even higher in the studies reported by Luna-More et al and Paterakos et al. (9.5 and 8.5 respectively) [(Luna-More et al. 2000), (Nassar et al. 2001), (Paterakos et al. 1999)]. Interestingly this high tendency for axillary nodal involvement is also described in relatively smaller tumors. It reaches 71% in T1 tumors (Luna-More et al. 2000). According to Walsh and Bleiweiss, two third of the IMPCa lesions with a pT1b and 74% of lesions with pT1a tumor stage had positive regional lymph nodes (Walsh and Bleiweiss 2001). Similarly, in a recent study, Vigniani et al also showed that these tumors were characterized by a significantly higher frequency of axillary metastases than that observed with invasive breast carcinoma NST (69% versus 47%), and by a large proportion of node positive cases involving four or more lymph nodes (56% versus 36%) (Vingiani et al. 2013).

As we pointed out in one of our previous publications on the topic, “it is possible that tumor foci with micropapillary growth pattern are a source of lymph node metastases, since examination of axillary contents revealed micropapillary differentiation in the lymph node tumor in most cases. It may be thus hypothesized that IMPCa represents an evolution of a subclone, acquiring a special morphology, and having a high propensity for regional lymphatic spread. This interpretation is further supported by the observation that foci of micropapillary growth were generally observed at the periphery (*i.e.*, invasive growth front) of the primary tumor. The mechanism by which IMPCa facilitates nodal metastases is, at this point, unclear” (Nassar et al. 2001).

The correlation between micropapillary morphology in breast cancer and an aggressive clinical behavior with worse survival is controversial. Paterakos et al., in their multivariate analysis of 21 cases of IMPCa failed to confirm that micropapillary phenotype predicted more aggressive clinical behaviour and a shortened disease

free or overall survival (Paterakos et al. 1999). In one of our previous studies we showed that although the overall outcome was worse for patients with the diagnosis of IMPCa in comparison to patients with invasive breast carcinoma NST, when matched for lymph node status however, survival appeared to be similar in both groups. We then postulated that “the high proportion of node positive patients with advanced metastatic tumor burden and skin involvement likely explains this difference and concluded that the outcome of a patient with IMPCa depends largely on staging parameters in a manner analogous to invasive breast carcinoma NST” (Nassar et al. 2001). This finding has been confirmed by Vigniani et al. In their study of 49 IMPCa cases with follow-up they showed that despite the unfavourable clinicopathologic characteristics of these tumors and the fact that they arise more often as locally advanced disease, the micropapillary morphology is not an independent risk factor for locoregional and distant disease recurrence or for overall survival (Vingiani et al. 2013).

Our study demonstrates that in our patient population and similar to other populations of patients affected with breast cancer, lymph nodes involvement is significantly higher in IMPCa than in NST with a comparable stage at presentation. In addition the risk of local and distant recurrence is much higher than that in NST also when matching for nodal status.

3.6. Pathogenesis of IMPCa

3.6.1. Previous observations

To date, the studies that attempted to explain the special morphology of IMPCa and its behavior have been able to show, using electron microscopy, special stains and immunostains, that the secretory surface of the neoplastic cells faces the stroma or more precisely the clear spaces between the cancer cell clusters and the stroma [(Luna-More et al. 1994), (Nassar et al. 2004)]. As we mentioned previously we demonstrated using immunohistochemistry that MUC1 glycoprotein, which is located in the apical surface of normal glandular cells, can be identified in IMPCa, in the stroma-facing surface of the neoplastic cells. Studies have shown that MUC1, because of its high molecular weight and chemical properties is believed, when highly expressed, to result in decreased cell to cell and cell to extracellular matrix as

well as contraction of collagen type I [(Hilkens et al. 1995), (Hudson et al. 2001), (Winterford et al. 1999), (Wesseling et al. 1995), (Wesseling et al. 1996)]. In light of these observations, one can assume that, by its localisation at the surface of the cell facing the stroma (reversed polarity) in IMPCa, MUC1 may be responsible for the detachment of the cells from the stroma and the appearance of clear spaces around the cell clusters, which is one of the defining morphologic characteristics of IMPCa. If this finding can to a certain extent explain the appearance of IMPCa, there is no clear explanation of how this special morphology correlates with an aggressive tumoral behavior.

3.6.2. Genomic analysis of IMPCa of the breast

Molecular and genetic studies have been done in an attempt to explain the characteristic morphology and aggressive behaviour of IMPCa. These studies were mainly conducted on tumors in breast. Thor et al. used comparative genomic hybridization on 16 cases of invasive micropapillary carcinomas and identified specific genomic patterns such as 8p losses, 8q gains and 17p and 16q losses much more frequently than in invasive carcinoma NST and invasive lobular carcinoma (Thor et al. 2002). A comparison done by Marchiò C et al between 24 IMPCa and a series of 48 grade and ER-matched invasive breast carcinoma NST revealed that high cyclin D1 expression, high proliferation rates, and MYC (8q24) amplification were significantly associated with the former (Maranchie et al. 2000). They also demonstrated a striking genetic and immunohistochemical similarity between pure IMPCas and those mixed with invasive breast carcinoma NST regardless of the amount of the invasive micropapillary component (Marchiò et al. 2009). This finding might explain the aggressive behaviour of tumors even with a minor micropapillary component. They showed in addition an amplification of the chromosome 8p11.2-p12 in four of 12 mixed tumors, a region that harbours interesting therapeutic target candidates, such as *FGFR1* and *PPAPDC1B* (Marchiò et al. 2009).

One of the most recent studies of the genetic alterations in IMPCa was published by Gruel et al. The authors report the following observations: 1) “unsupervised analysis of genomic data distinguishes two IMPCa subsets, the first exhibiting a significant increase in 16p gains (71%), and the other characterised by a high frequency of 8q (35%), 17q (20% to 46%) and 20q (23% to 30%) amplifications and 17p loss (74%)”

2) “compared to invasive carcinoma NST, IMPCa exhibits specific loss of the 6q16-q22 region (45%), which is associated with downregulation of *FOXO3* and *SEC63* gene expression” and 3) “by using whole-exome sequencing combined with RNA sequencing of these tumors they identified in rare cases somatic mutations in genes involved in polarity, *DNAH9* and *FMN2* (8% and 2%, respectively) or ciliogenesis, *BBS12* and *BBS9* (2% each) or genes coding for endoplasmic reticulum protein, *HSP90B1* and *SPTLC3* (2% each) and cytoskeleton, *UBR4* and *PTPN21* (2% each), regardless of the genomic subset” (Gruel et al. 2014). These genetic alterations alone cannot explain the pattern of IMPCa and its behavior since they were not observed in all cases. Therefore the authors raise the possibility that other biological alterations (for example, epigenetic modifications, stromal alterations) could also play a significant role in this process. In another study Natrajan et al demonstrated that IMPCa is not defined by highly recurrent mutations or by recurrent fusion gene (Natrajan et al. 2014).

In the current study we performed NGS on six pure IMPCa of the breast. Most of these cases did not show any mutation of the 50 genes included in our panel. In two cases (one third of the total), mutations of the TP53 and PIK3CA genes were detected, each in one case. A mutation of the MET gene (*MET*-T1010I) was seen in the tumor with TP53 gene mutation. The presence of activating point mutations in IMPCa of the breast was also addressed in a study by Flatley et al. The authors identified Hotspot point mutations in 35% of IMPCa including PIK3CA exons 7, 9 und 20 Hotspots as well as the AKT1 plekstrin homology domain mutation (E17K); mutations in TP53 and KRAS were each found in 5% of the cases. They concluded that the rate of PIK3CA mutation in their series was similar to that described for invasive carcinoma NST but noted that there might be enrichment of AKT mutations in IMPCa (Flatley et al. 2013).

In breast cancer, TP53 gene mutations are the second most commonly observed genetic alterations -mutations in the PIK3CA gene are the most frequent- being detected in 30% of the cases. Their distribution correlates significantly with the molecular subtype; they are found in 26% of luminal A and B tumors, 50% of HER2 and 88% of basal like subtypes (Gui et al. 2016). In the two latter subtypes the mutations are usually complex whereas base pair substitutions occur in the luminal type similar to that seen in our IMPCa series. On the other hand, *MET*-T1010I

germline mutation is very rare in breast cancer occurring in approximately 2% of patients with metastatic breast cancer. It has been shown that “overexpression of wild type *MET* (*MET*-WT) as well as expression of *MET*-T1010I increases colony formation, cell migration and invasion *in-vitro* and tumor growth *in-vivo*. Moreover, in comparison to *MET*-WT, *MET*-T1010I appears to selectively impact cell invasion and therefore, according to the authors, it should be considered in the clinical trials of MET inhibitors (Liu et al. 2015). In light of these observations, *MET*-T1010I mutation in one of our IMPCa cases is of interest in potentially explaining the aggressive behavior of IMPCa since it is mostly seen in metastatic breast cancer and in offering targeted therapy to patients with tumors harboring this mutation. The numbers in our series are too small to be able to draw definite conclusions. It is therefore important to specifically look for this mutation in a larger series of IMPCa of the breast.

4. Summary

Invasive micropapillary morphology is associated with clinically meaningful staging and outcome implications in patients with breast cancer. The characteristic micropapillary morphology is seen focally in the tumor in most cases. In contrast to other special subtypes of breast cancer, any proportion of invasive micropapillary growth confers to the tumor a “special” aggressive behaviour characterised by lymphovascular invasion, high propensity for lymph node metastases and high stage at presentation. At the molecular and immunohistochemical level, tumors with mixed invasive micropapillary and invasive carcinoma NST display striking similarities to those with pure invasive micropapillary carcinomas. Moreover, these tumors are classified in most cases in the category of luminal B molecular subtype.

Invasive micropapillary carcinoma has been described in other organ systems, although less frequently as in breast. In the urinary tract, gastrointestinal tract, lungs and salivary glands, this tumor pattern is in general admixed with a more conventional tumor growth pattern, and same as in breast, presents as a locally advanced disease and is associated with an aggressive behaviour.

To date there are few molecular studies performed on IMPCa of the breast. None of them shows characteristic genetic alteration that could explain the special morphology of the tumor or its aggressive course. In other terms, IMPCa is not defined by highly recurrent specific mutations or fusion genes. In few cases reported in the literature as well as in the study that we presented, hotspot point mutations of the PIK3CA gene, and the TP53 gene were identified however at a rate similar to that seen in invasive breast carcinoma NST. In addition we saw in one of our IMPCa cases a *MET*-T1010I germline mutation, which is usually very rare in breast cancer, occurring in 2% of patients with metastatic breast carcinoma. Because of the therapeutic consequences (with MET inhibitors) we suggest a more extensive testing of the MET gene in this aggressive type of breast cancer.

Zusammenfassung

Mikropapilläre Morphologie des invasiven Mammakarzinoms hat bedeutende Konsequenzen für Brustkrebs Patientinnen, da sie Einfluss auf Staging und Prognose nehmen. In den meisten Fällen ist das mikropapilläre Wuchsmuster ein Teil des Tumors und tritt mit anderen Subtypen des Mammakarzinoms gemischt auf. Im Gegenteil zu anderen speziellen Typen ist jeder mikropapilläre Anteil im Tumor mit einem aggressiveren Verhalten assoziiert, beispielweise Lymphangioinvasion, Lymphknotenmetastasen und fortgeschrittenem Tumorstadium. Auf molekularer und immunhistochemischer Ebene verhalten sich die gemischten IMPCa und NST Tumoren verglichen mit den reinen IMPCa sehr ähnlich. In den meisten Fällen gehören diese Tumoren zu den molekularen Luminal B Syptypen.

IMPCa wurden bereits in anderen Organen beschrieben, allerdings nicht so häufig wie in der Mamma. Bei Neoplasien des Harn- und Gastrointestinaltrakts, der Lungen oder der Speicheldrüsen tritt das mikropapilläre Wuchsmuster auch mit *konventionellen Karzinomtypen* gemischt auf. Ebenso wie bei Mammakarzinomen präsentieren sich diese Tumoren in lokal fortgeschrittenem Tumorstadium und sind mit einem aggressiveren Verhalten assoziiert.

Bis jetzt sind nur wenige molekulare Studien zu IMPCa der Mamma durchgeführt worden. Keine dieser Studien konnte bislang weder die spezielle Morphologie noch das aggressive Verhalten erklären. Zudem kann das IMPCa der Mamma den Studien zufolge nicht über hoch *repetitive* spezifische Mutationen oder Genfusionen definiert werden.

In wenigen Fällen sowie in unserer Studie wurden Punktmutationen der PIK3CA- und TP53-Gene gefunden, doch mit der gleichen Häufigkeit wie in NST Mammakarzinomen. Zusätzlich konnten wir eine MET-T1010I Keimbahnmutation beweisen, welche normalerweise sehr selten in Mammakarzinomen vorkommt und zwar in durchschnittlich 2% der metastasierten Tumoren. Aufgrund der daraus resultierenden Therapiemöglichkeiten (MET-Inhibitoren) wäre ein ausführlicheres Testen in Hinblick auf das MET-Gen in IMPCa der Mamma ratsam.

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6. Statement of the author's role in the study

The design of the study, the collection of most of the clinical and pathological data, the interpretation of the data, including clinical, pathological, and immunohistochemical, the literature search and the writing of the dissertation were entirely done by me.

Prof. Dr. F. Fend supervised the work.

The technical work (Tissue microarrays and immunohistochemistry) were done by our technicians in the pathology laboratory of the Universitätsklinikum Tübingen.

The performance and interpretation of the NGS was done by Dr. Irina Bonzheim.

Dr. Zeid Bittar participated in the interpretation of controversial immunohistochemical data and the collection of 20% of the clinical and pathological data.

Since I have previously worked on other series of IMPCa that ended in publications, some similarities are to be noticed between statements in the discussion here and in those of the already published papers. One figure (Figure 4) is also taken from one of these publications. This information is referred to in the sections of discussion and Figures.

7. Acknowledgements

Part of the work (TMA, immunohistochemichal staining) was performed with the support of a grant from the Alexander von Humboldt Foundation.

I would like to thank Dr. Irina Bonzheim for her help in the performance and interpretation of the NGS as well as her assistance in the statistical analysis.

I also thank Dr. Zeid Bittar for his help in collecting part of the data and in interpreting some of the controversial immunostains as a second pathologist.

I would like to thank Prof. Falko Fend for his mentorship and his critical review of the work.