Diplomarbeit

Expression Profile of Metalloproteases in Neuroendocrine Tumour Cell Lines

Expressionsprofil von Metalloproteasen in Neuroendokrinen Tumor Zelllinien

eingereicht von

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zur Erlangung des akademischen Grades

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(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt am

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Graz, am 10.11.2014

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Acknowledgments

Vor allen möchte ich meinen Eltern, Peter und Elfi Krismer, für Ihre Liebe, Aufopferung, Ermutigung und immer währende Unterstützung, von ganzem Herzen danken. Natürlich auch für den jahrelangen finanziellen Beistand, ohne den mein Studium nicht möglich gewesen wäre. Danke dass Ihr mir immer zur Seite gestanden seit Mama und Papa!

Ebenso möchte ich Frau Mag. Dr. Nassim Ghaffari Tabrizi-Wizsy für die herzliche Betreuung und Ihr außerordentliches Engagement danken. Danke für die vielen Stunden der angeregten Diskussionen, guten Ratschläge, aufbauenden Worte, und für den Einblick in die spannende Welt der Tumorforschung.

Herrn Prof. Anton Sadjak danke ich, für meine Zeit als Studienassistent und Diplomand, für das Bereitstellen der Infrastruktur, sowie die fließende Integration ins hauseigene Team und für die gute Stimmung am Institut.

Frau Prof. Roswitha Pfragner danke ich, für das Bereitstellen der Zell Kulturen, sowie für die Hilfe bei der Literaturrecherche, und für die spannenden Einblicke in die Welt der Zell Kultur.

Den vielen fleißigen Händen und offenen Türen am Institut für Pathophysiologie sei ebenso herzlich gedankt.

Für die produktiven und weniger produktiven Lernpausen, sei den Herren Bergenz, Kranabitl, Schinagel, Schmied, Regele und Zilke Dank auszusprechen.

Zwei besonderen Menschen gilt an dieser Stelle noch mehr als nur ein Dankeschön. Meiner Schwester Viola, die all meine Flausen über die Jahre ertragen hat und mir mit Witz und Charme immer zur Seite gestanden ist, und meiner Freundin Annabel die mir mit Ihrer herzlichen Art und Ihrem sonnigen Gemüt seit jeher ein Lächeln auf die Lippen zu zaubern vermag.

Abstract

Neuroendocrine Tumours (NETs) are indolent neoplasms occurring all over the body. Despite their typically slow growth, they are primarily detected when already metastasized. Since NETs are relatively insensitive to radiation therapy as well as chemotherapy, new approaches for novel treatments are urgently needed. Overexpressed Metalloproteases have shown to play a crucial role in the process of tumour progression and metastasis and might therefore be considered valuable therapeutic targets. With a rising incidence every year the relevance of Neuroendocrine Tumours will be considered equal to testicular cancer or myeloma in 2015.

Aims

Since Metalloproteases are not well studied in NETs, the aim of this study was to explore expression profiles of the currently known A Disintegrin and Metalloproteases (ADAMs) and Matrix Metalloproteases (MMPs) for 10 Medullary Thyroid Carcinoma (MTC) and 2 Small Intestine NET (SI-NET) cell lines.

Methods

The expression analysis was performed at Ribonucleic Acid (RNA) level. Therefore total RNA was isolated from MTC cell lines BOJO, GRS-IV, GRS-V, HEVE-II, MTC-SK, OEE-III, RARE, SHER-1, SINJ, TT and SI-NET cell lines P-STS and KRJ-I, following Reverse Transcription and Polymerase Chain Reaction (RT-PCR). The design of appropriate PCR primers of human MMPs and ADAMs was also a major part of this work.

Results and conclusion

We could create expression profiles of the currently known ADAMs and MMPs in 10 MTC and 2 SI-NET cell lines. Summarized our findings report a generally high protease expression for ADAMs, with ADAM 10, 15, 17, 21 and 22 expressed throughout all cell lines. Contrary, MMP expression was rather low, with only MMP 7 present in most NET cell lines. In accordance with the current literature we proclaim closer investigation of ADAM 10, 15 and 17 as putative biomarkers for MTC. However, further investigation is needed to ascertain the utility of Metalloproteases as a target for anti-neoplastic agents, or as prognostic biomarkers for NETs.

Zusammenfassung

Neuroendokrine Tumore (NET) vereinen eine Gruppe von relativ seltenen, schmerzlosen Neoplasien, welche überall im menschlichen Körper auftreten können. Abgesehen von ihrem typisch langsamen Wachstum, werden NET meist erst dann entdeckt, wenn sie bereits metastasiert haben. Aufgrund ihres schlechten Ansprechens auf Radio- und Chemotherapie und ihrer steigenden Inzidenz in den letzten Jahren, ist die Nachfrage nach neuen Therapiekonzepten stark angestiegen. Die Überexpression von Metalloproteasen konnte in verschiedenen Studien in direkten Zusammenhang mit Tumor Progression und Metastasierung gebracht werden, was die Hemmung dieser Enzyme zu aussichtsreichen, potentiell therapeutischen Mittel macht.

Ziele

Da die Expression von Metalloproteasen in Neuroendokrinen Tumoren noch relativ unerforscht ist, bestand das Ziel unsrer Arbeit darin, die Expressionsprofile der zurzeit bekannten A Disintegrin und Metalloproteasen (ADAMs) und Matrix Metalloproteasen (MMPs) in 10 Medullären Schilddrüsenkarzinom- (MTCs) und 2 Dünndarmkarzinoid (SI-NET) Zelllinien zu erforschen.

Methoden

Die Expressionsanalysen wurden auf Ribonukleinsäure (RNA) Niveau ausgeführt. Für diesen Zweck wurde RNA aus den Medullären Schilddrüsenkarzinomlinien BOJO, GRS-IV, GRS-V, HEVE-II, MTC-SK, OEE-III, RARE, SHER-1, SINJ, TT, und den beiden Dünndarmkarzinoid Zelllinien P-STS und KRJ-I isoliert. Anschließend wurde Reverse Transkriptase - Polymerase Kettenreaktion (RT-PCR) durchgeführt. Das Erstellen passender Primer Paare, für alle humanen MMPs und ADAMs, war ebenso ein großer Teil dieser Arbeit.

Ergebnisse und Schlussfolgerung

Wir konnten Expressionsprofile für die gegenwärtig bekannten ADAMs und MMPs in 10 MTC und 2 SI-NET Zelllinien erstellen. Unsere Untersuchungen beschreiben eine generell hohe Expression aller ADAMs, von denen ADAM 10, 15, 17, 21 und 22 in allen Zelllinien vorkommen. Gegenteilig, konnten wir eine nur sehr geringe MMP Expression nachweisen. Lediglich MMP 7 war in den meisten Neuroendokrinen Zelllinien präsent. Unsere Ergebnisse, betreffend der ADAM Expression, stehen in Einklang mit der aktuellen Literatur. Wir proklamieren ADAM 10, 15 und 17 als mögliche Biomarker für Medulläres Schilddrüsenkarzinom und stellen fest, dass weitere Nachforschungen nötig sein werden um den Nutzen von Metalloproteasen in Tumortherapie und als prognostische Marker zu evaluieren.

Abbrevations

ADAMDEC1	ADAM-like Decysin Gene
ADAMs	A Disintegrin and Metalloproteases
ADAMTS	ADAMs with Thrombospondin Motifs
APUD	Amine Precursor Uptake and Carboxylation
ATCC	American Cell Culture Collection
BG	Background
BPs	Base pairs
BRAF	Proto Oncogene B-Raf
CEA	Carcinoembryonic antigen
CHGA	Chromogranin A
CHGB	Chromogranin B
CO_2	Carbondioxide
CUX 1	Cut-like Homeobox 1
DEPC	Diethylpyrocarbonate
DNA	Desoxyribonucleic Acid
DNES	Diffuse Neuroendocrine System
DSMO	Dimethylsulfoxid
ECC	Enterochromaffin Cell
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial Mesenchymal Transition
ENO1	Enolase 1
ENO2	Enolase 2
EtBr	Ethidium bromide
EtOH	Alcohol
FBS	Fetal Bovine Serum
FMTC	Familial Medullary Thyroid Carcinoma
FNA	Fine Needle Aspiration
GEP-NETs	Gastroenteropancreatic NETs
GI	Gastrointestinal
H_2O	Water
HBSS	Hanks Balanced Salt Solution
HPX	Hemopexin
In Silico PCR	In Silico Polymerase Chain Reaction
L30	Ribosomal Protein L30
M2BP	Lectin, Galactoside-binding, soluble, 3 Binding Protein
MEN	Multiple Endocrine Neoplasia
MEPs	Metalloendopeptidases
MET	Hepatocyte Growth Factor Receptor
MM	Mastermix
MMPs	Matrix Metalloproteases
MP	Metalloproteases
MTC	Medullary Thyroid Carcinoma
NCI	National Cancer Institute
NETs	Neuroendocrine Tumours

NFH_2O	Nuclease Free Water
PGs	Proteoglycanes
pH	Decimal of the reciprocal of the hydrogen ion activity
RECK	Reversion-Inducing Cysteine-Rich Protein with Kazal Motifs
RET	RET proto oncogene
RNA	Ribonucleic Acid
RNase	Ribonuclease
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SCG2	Secretogranin II
SCG3	Secretogranin III
SCG5	Secretogranin V
SEER	Surveillance, Epidemiology and End Results
SI-NETs	Small Intestine Neuroendocrine Tumour cell lines
SOP	Standard Operating Procedure
SVMPs	Snake Venom Metalloproteases
SYPb	Synaptophysin b
TACE	Tumour Necrosis Factor alpha Converting Enzyme
TAE	Tris Acetate EDTA
TIMPS	Tissue Inhibitor of Metalloproteases
TKI	Tyrosin Kinase Receptor inhibitors
TNF- α	Tumour Necrosis Factor α
TNM	Tumour, Node, Metastasis Staging system
VEGF	Vascular Endothelian Growth Factor

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1.1 Overview

Neuroendocrine tumours (NETs) are relative rare, typically slow growing, indolent neoplasms that derive from the neuroendocrine cell system, which is spread all over the body. Despite their different origins, they share several uniting histopathological features in morphology, cytology (small – medium cells) or growth pattern (nests) (ed. Yao *et al.* 2011). Usually NETs stay undetected over many years, because most of them do not show any symptoms, lack sufficient methods of detection or aware physicians. But sometimes they can present themselves more rapidly, by producing hormones, or through aggressive growth and obstruction followed by clinical symptoms (Öberg & Castellano 2011). Though they are known for over 100 years, they are still challenging to diagnose and treat. This reason and a late significant increase, make them a valuable target for further investigation (ed. Yao *et al.* 2011). With a rising incidence every year the relevance of neuroendocrine tumours will be considered equal to testicular cancer or myeloma in 2015 (Frilling *et al.* 2012).

1.2 History

In 1867, Theodore Langhans firstly described a peculiar neoplasm of the small gut while performing an autopsy. The unusual lesion presented itself as a very sharp lined, mushroom-shaped, intraluminal growing, non-invasive tumour. While he focused on the histologic appearance of the nest forming, poorly differentiated, glandular tissue, it took 21 more years until Otto Lurbach identified it as carcinoma. After he considered 35 similar cases of intestinal tumours, he was still

not convinced if any of them were truly malignant. The riddle of the unique lesion continued and neither William Bramwell Ransom, who described the malignancy to be only local, nor A. Notthafft who started speaking of “beginning carcinomas” could offer a satisfying characterization (Modlin *et al.* 2004).

German pathologist Siegfried Oberndorfer noted specific trends of these tumours and summarized them:

- Small lesions in patients with multiple tumours
- Very slow growth
- Undifferentiated tissue nearby & might form glands
- Possibility of invasion
- No metastasis

While the histology of the lesion appeared malignant, its nature and clinical features could not apply to standard carcinoma. Therefore Oberndorfer offered a new term, calling it *Karzinoid*, signifying a carcinoma like lesion (Oberndorfer S., 1907). Although he revised his characterization of a harmless tumour years later and acknowledged that there is possible metastasis, his work still remains the most important piece in pioneer carcinoid research (Modlin *et al.* 2004).
5 years earlier in 1902, W. M. Bayliss and E. H. Starling identified the gut as an endocrine organ. In the late 19th century the origin of the carcinoid cell, the enterochromaffin cell (ECC) was discovered, without knowing its role and function (Modlin *et al.* 2004). Austrian physician Fred Lembeck was the first to investigate that ECC built up and secreted serotonin, a biogenic amine (Lembeck 1953). Through staining methods F. Feyrter discovered more of these clear cells (*Helle Zellen*) in all body tissues, but especially in the gut (Modlin *et al.* 2004) Pearse noticed, that all of these endocrine cells had similar cytochemical features, structure and behaviour (hormone production). He characterized them as APUD cells (Amine Precursor Uptake and Carboxylation cells) and constructed the *1st Neuroendocrine Concept (Pearse 1979)*. Later on these cells were inferred to as the Diffuse Neuroendocrine System (DNES), because they would occur from every endocrine organ (endoderm) (Langely 1994). Because of the disproven

thesis that all neuroendocrine tumours derive from the neural crest, discussions were made to change the name into endocrine tumours. Further research supported the fact that they would arise from almost any organ. But even though the WHO still recommends them to be described as neuroendocrine tumours (Klimstra *et al.* 2010).

1.3 Classification

Classification has been a very controversial topic in NET history since the beginning. Whether they should be arranged by history, embryologic origin, histology or function, disagreement below researchers has led to multiple classifications systems, different terms and confusion (Modlin *et al.* 2004). Although NETs share many common features, an overall standard without the implication of their primary sites seemed impossible, due to the fact that some neuroendocrine features are directly bound to the specific organ (Klimstra *et al.* 2010). Troubling, according to Yao J. C., is yet the existence and use of overpassed nomenclatures, concepts a century old and also the fact that, although most of the neuroendocrine tumours derive from the gastrointestinal tract, the commonly used classification systems have been designed from studies done in thoracic tumours (ed. Yao et al. 2011). Most nomenclatures have distinguished an assignment between well und poorly differentiated tumours in context with tumour grading (aggressiveness of the tumour). While well-differentiated tumours correlate to intermediate or low grade, poorly differentiated tumours always present a high grading, which defines them as neuroendocrine carcinoma. The latest WHO classification for thymus, lung and gastroenteropancreatic NETs (GEP – NETs) is shown in Figure 1. and 2. The WHO 2010 concept, together with the Tumour Node Metastases (TNM) staging of the The American Joint Committee on Cancer is to be considered the current state of the art for clinical practice. It is based on differentiation, origin of the tumour, clinical symptoms (Klimstra *et al.* 2010) and histological grading (KI67 index, mitotic rate) (Yang et al. 2013).

Grade	Lung and Thymus (WHO)[34]	GEP-NETs (WHO 2010)[3]
Low grade	Carcinoid tumor	Neuroendocrine neoplasm, grade 1
Intermediate grade	Atypical carcinoid tumor	Neuroendocrine neoplasm, grade 2
High grade	Small cell carcinoma	Neuroendocrine carcinoma, grade 3, small cell carcinoma
	Large cell neuroendocrine carcinoma	Neuroendocrine carcinoma, grade 3, large cell neuroendocrine carcinoma

Figure 1. Lung, thymus and GEP NET grading I (Klimstra *et al.* 2010)

Grade	Lung and Thymus (WHO)[34]	GEP-NETs (ENETS, WHO)[3,28,29]
Low grade	<2 mitoses / 10 hpf AND no necrosis	<2 mitoses / 10 hpf AND <3% Ki67 index
Intermediate grade	2–10 mitoses / 10 hpf OR foci of necrosis	2–20 mitoses / 10 hpf OR 3%–20% Ki67 index
High grade	>10 mitoses / 10 hpf	>20 mitoses / 10 hpf OR >20% Ki67 index

Figure 2. Lung, thymus and GEP NET grading II (Klimstra *et al.* 2010)

1.4 Epidemiology

According to the leading institutes namely, the National Cancer Institute (NCI) and the Surveillance, Epidemiology and End Results (SEER), a significant advance (1973 – 2005) from 1.1 to 6.2 / 100.000 individuals (+ 520%), implicating an increase of 5.8% each year has been noted (Lawrence *et al.* 2011a). Although better diagnostics and more awareness of physicians might add a big part to that number, the impact of this disease is speculated to have an equal relevance as esophageal-, testicular cancer, or myeloma in 2015. In terms of occurrence NETs are predominantly found in in the gastrointestinal (GI) tract (60.9% of NETs), followed by the bronchopulmonary system (27,4%), and pancreas (1%).

The hepato-biliary system, testes and ovaries, thyroid and other organs bearing neuroendocrine cells are additional rare sites of NET occurrence. Furthermore the distribution in the GI tract is led by the small intestine (30.8%), followed by rectum (26.3%), colon (17.6%), stomach (8.9%) and appendix (5.7%) (Frilling *et al.* 2012).

1.5 Etiology and Risk Factors

Alcohol consumption and cigarette smoking showed no significant association with NETs. However a link between nutritional or occupational factors and neuroendocrine tumours could be found. Men occupied in metal and footwear, as well as woman in food and beverages, showed compelling increase of NET especially within long term employment (>25 years). Furthermore a specific risk for tumours of the small intestine occurred, independently of gender aspects, in bookkeepers, shoemakers, machine filters and construction painters. Nutrition studies showed that exposure to high saturated fat was relevant to create an increase in neuroendocrine tumours of the small intestine. Fibre diet could reduce the risk of small bowel disease in about 40%. Chronic medical conditions like diabetes mellitus showed an important relationship to gastric NETs. The genetic impact on NETs has still to be examined more deeply and in higher numbers. But a genetic etiology has been stated for ancestral syndromes, like tuberculosis, sclerosis, multiple endocrine neoplasia typ 1 (MEN-1), non polyposis colon cancer, neurofibromatosis typ1, and von Hippel-Lindau syndrome (ed. Yao *et al.* 2011).

1.6 Location

Included are tumours of the GI tract, lungs, pancreas, thymus and of more infrequent regions as thyroid, heart, ear, testes and ovaries. NETs of the gastrointestinal tract have been furthermore separated into endocrine pancreatic and carcinoids tumours (Öberg and Castellano 2011).

Out of tradition, another differentiation into stomach, duodenum, thymus and lung, representing the foregut, jejunum, ileum, appendix and colon ascendens, referred as the midgut, and the rest of the colon, plus rectum as hindgut is commonly found (Modlin *et al.* 2004).

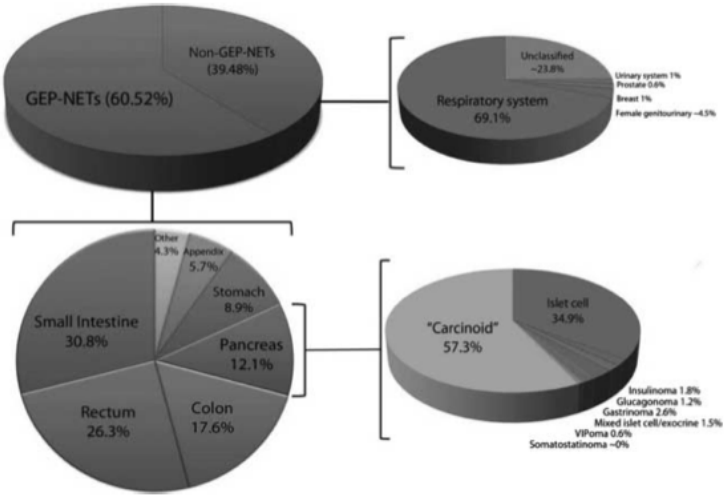

Figure 3. NET classification (Frilliing et al 2012)

1.7 Therapy

NET treatment is based on tumour grading levels. While G3 (high grade) tumours get treated with chemotherapy (Cisplatinum-Etoposide), the only existing curative option for G1 (low) and G2 (intermediate grade) tumours is surgery (Dong *et al.* 2012). Therefore alternative therapeutic targets are highly wanted. Several novel agents have been investigated, targeting angiogenesis, growth factor receptors or molecular pathways. Although functional agents have been found for G1/G2 graded tumours, chemotherapy (Streptozotocin, 5 FU) still outruns the newer agents due to a response rate of 30 – 40% vs. an efficiency of 8 – 10% for novel agents as first line alternative treatment after surgery. In G3 tumours Everolimus and Sunitinib suggest beneficial therapeutic outcome but the effect onto clinic impact is too early to point out. The detailed suggested therapeutic model is shown in Figure 4. (Öberg 2012).

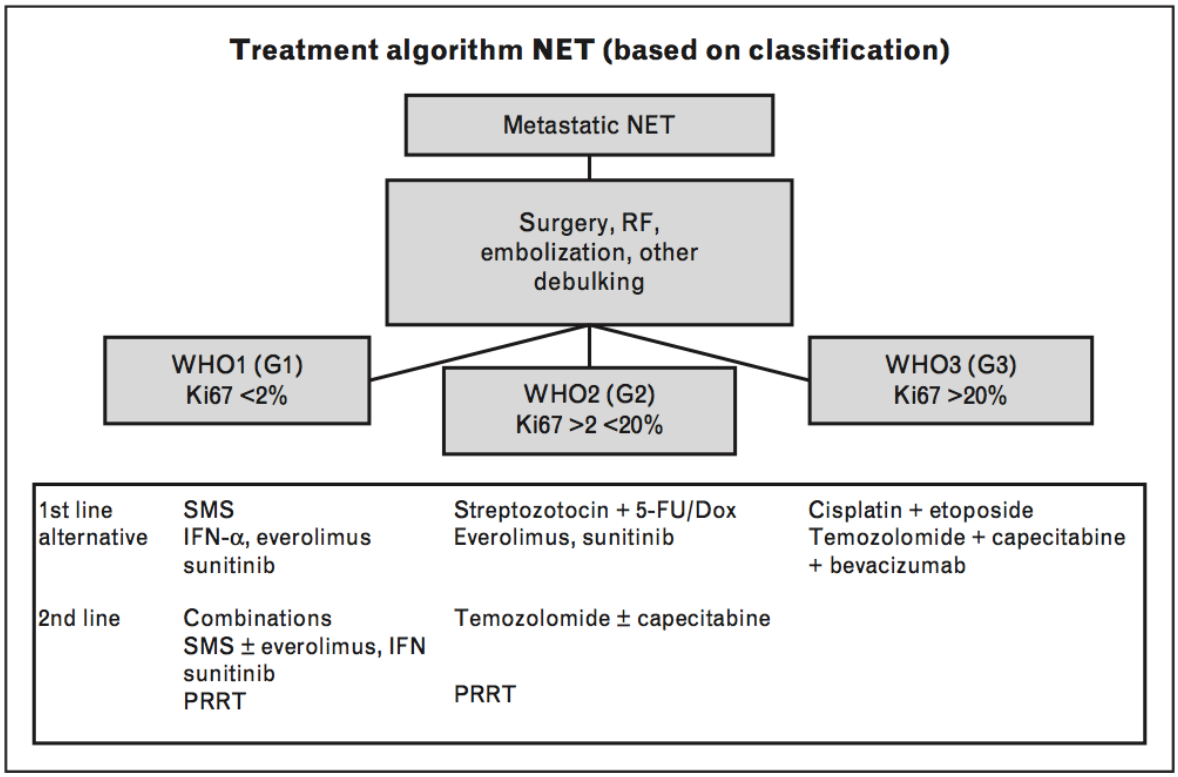

Figure 4. Suggested NET treatment algorithm (Öberg 2012)

1.8 Medullary Thyroid Carcinoma

1.8.1 Introduction

Thyroid cancer represents the most common malignant neoplasm in endocrine organs. Classified into 4 subtypes, (follicular, papillary, anaplastic and medullary thyroid carcinoma) the major part of these tumours derives from a thyrocytic origin (Schneider and Chen, 2013). Medullary Thyroid Carcinoma (MTC) represents around 3 – 4 % of thyroid malignancies (Roy *et al.* 2013).

MTCs arise from the calcitonin producing parafollicular C-cells. These cells derive from the neural crest, assigning them to neuroendocrine tumours (NETs) within the thyroid gland. In 75% MTC occur sporadic, whereas in 25% they appear in a

hereditary manner, either isolated as FMTC (Familial Medullary Thyroid Cancer) or as Multiple Endocrine Neoplasia (MEN2A, 2B) (Cerrato *et al.* 2009).

In contrary to the well -differentiated and -treatable thyroid malignancies (follicular and papillary cancers), medullary thyroid carcinomas come with a poor prognosis and altered incidence for metastasis (Ganeshan *et al.* 2013).

1.8.2 Epidemiology

Female patients around 50 – 60 years represent the typical MTC patient. The hereditary forms arise in a much younger age. MEN2A and FMTC occur more often in the third life decade, whereas MEN2B is found in patients under the age of 20 (Roy *et al.* 2013).

1.8.3 Clinical Features

Lymph node enlargement (35-50%) and/or a palpable neck mass are usually the first clinical signs for a thyroid malignancy. Medullary thyroid carcinoma is especially located between the upper third and lower two thirds of the thyroid gland, although C cells exist anywhere in the organ. MTCs occur mostly in the posterior section of the gland and can cause, because of the proximity to tracheal tube and esophagus, swallow difficulties, croakiness or respiratory problems. If the tumour produces high calcitonin levels, other symptoms, like diarrhoea, weight loss might occur (Roy *et al.* 2013).

1.8.4 Diagnosis

Secondary to clinical presentation, histopathology *via* fine needle aspiration (FNA) secures the diagnosis of MTC. The accuracy of the tissue extraction lies between 50 – 80 %. The absence of thyroid follicles and presence of stromal amyloid is the

classic histology pattern presented by this tumour. In ambiguous situations immunohistochemical staining (calcitonin, chromogranin, carcinoembryonic antigen (CEA), synaptophysin or other neuroendocrine markers) can facilitate the diagnosis. Also elevated calcitonin is an excellent MTC predictor, because it occurs in 98-99% of the time. Basal levels higher than 20pg/ml are defined as suspicious. For hereditary forms genetic screening of family members is highly recommended (Ganeshan *et al.* 2013, Roy *et al.* 2013).

1.8.5 Therapy

1.8.5.1 Surgical Treatment

Surgical intervention is considered the most beneficial way of treatment, because MTC is relatively insensitive to radiation therapy and chemotherapy. Secondary to tumour excision (total thyroidectomy), bilateral central neck dissection is performed. If a *RET proto oncogene* mutation is identified, prophylactic thyroidectomy is advised to prohibit MTC development (Ganeshan *et al.* 2013, Roy et al. 2013).

1.8.5.2 Medical Treatment

Patients treated with chemotherapy (5 –Fluorouracil, Darcabazine, Capecitabine or Doxorubicin) response in 24% to 29% to the therapy. Newer agents like Tyrokinase Receptor Inhibitors (TKIs) could show response rates up to 50% in clinical trials (phase 2). Targets of those medications are the Vascular Endothelian Growth Factor (VEGF), proto oncogene B-Raf (BRAF), Epidermal Growth Factor Receptor (EGFR), Hepatocyte Growth Factor Receptor (MET) and proto oncogene RET (Roy *et al.* 2013).

1.8.5.3 Prognosis

The 10-year survival rate is around 75-85% and even higher if the lesion is limited to the thyroid gland (up to 95%). If there are already metastases the 10-year survival drops to 20 - 40%. Metastasis is found in 13% of all initial diagnosis (Roy *et al.* 2013, [1]).

Stage I (T1, N0, M0): <2 cm	**95%**
Stage II (T2, N0, M0): 2 to 4 cm	
Stage III (T3, N0, M0 or T1 to T3, N1a, M0) >4cm	**75%**
Stage IVA (T4a, N0 to N1a, M0 or T1 to T4, N1b, M0)	
Stage IVB (T4b, any N, M0)	
Stage IVC (any T, any N, **M1**)	**20-40%**

Figure 5. TNM staging of medullary thyroid carcinoma [1]

1.9 Cancer and Proteases

During the initial step of the metastatic cascade, the epithelial mesenchymal transition (EMT), primary tumour cells get transformed and acquire the ability to invade and form secondary metastatic sites (Chaffer and Weinberg 2011). Several proteolytic enzymes were found to be overexpressed in this process, correlating directly to a malignant turnover in tumour behaviour and a breakdown of the natural barrier, e.g. the Extracellular Matrix (ECM). Beside 4 well known protease families that manage to degrade ECM components (e.g. Matrix Metalloproteases (MMPs), serine-, cysteine-, and aspartatic proteases) (Schröpfer et al. 2010) also some individual A Disintegrin and Metalloproteases (ADAMs 10, 13 and 15) share these proteolytic features and contribute to the invasion-metastatic cascade (Seals and Courtneidge 2003).

2. Metalloproteases

Metalloproteases (MPs) - also known as Metalloproteinases or Metalloendopeptidases - are hydrolytic enzymes commonly found in all species (Gomis-Rüth 2003). Because of the zinc atom that is integrated in their active site the carry the name “Metallo”proteases (Ramussen and McCann, 1997). These zinc dependent enzymes are involved in tissue development, remodelling and maintenance. They occur as inactive zymogens that contribute to many regulatory processes, such as: cell-cell interactions, hormone homeostasis, blood pressure control and many more. They are controlled at a transcriptional level and get either activated or inactivated by cleavage. They can also activate/deactivate other enzymes or DNA (Desribonucleic acid) repressors. Unregulated activation can lead to neurological and cardiovascular disorders and plays a role in inflammation and tissue destruction, as well as in tumour progression, invasion and metastasis. MPs also contribute to zoonotic and anthroponotic diseases, as they provoke hemorrhage after snake bites and can cause meningitis, gas gangrene or tetanus botulism, during bacterial infections (Gomis-Rüth 2003).

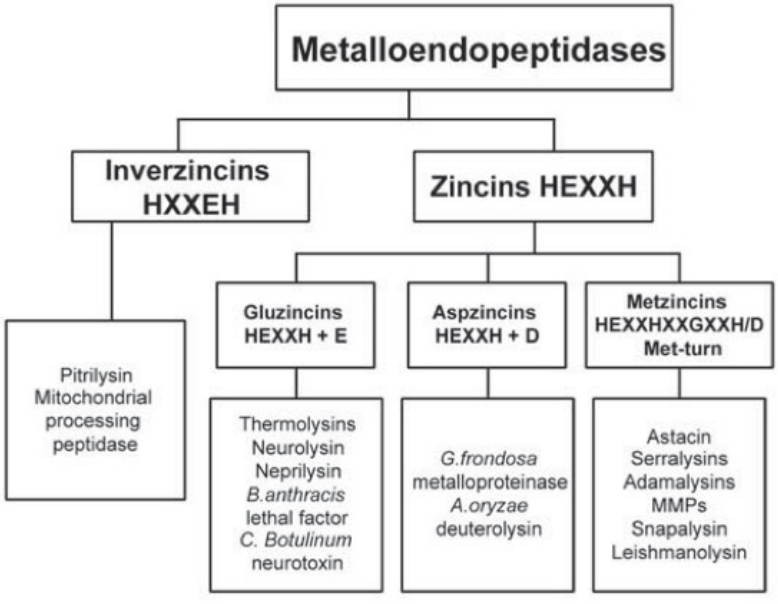

Figure 6. MP classification (Gomis-Rüth 2003)

2.1 Matrix Metalloproteases

2.1.1 Overview

Matrix Metalloproteases (MMPs) represent a zinc dependent endopeptidase family (Hagemann *et al.* 2010) of structural and functional related zymogens (Schröpfer et al. 2010). MMPs are part of the Metzincin group (Hooper 1994). This division is further separated into Serralysins, Astacins, Adamalysins and Matrixins, including Matrix Metalloproteases (Stöcker *et al.* 1995). They are listed in appearance of their discovery (MMP1 – 28) as a current collective of 23 different genes in humans. Their capability of degrading ECM components derives from an imbalance in a tightly regulated cascade of activation and inhibition MMPs have shown to play crucial parts in physiological processes like tissue remodelling and development as well as in pathological events such as tumour progression, cardiovascular diseases and rheumatoid arthritis (Hagemann *et al.* 2012). ECM proteolysis shows but two sides. One is degrading proteins and destroying structural cell components paving the way for tumour invasion and the other is, cleaving new functional proteins due to growth factor activation into conditionally accessible signalling molecules (Page-McCaw *et al.* 2007). This bivalent behaviour makes them an utterly interesting target for an anti - neoplastic approach.

2.1.2 History

MMPs, Matrixins or Vertebrate Collagenases were discovered in 1962 during trials investigating *Xenopus* development (tail resorption) and could be identified as the major processor of ECM components (Tallant *et al.* 2010). 13 years after Lapiere and Gross´s description of this collagenolytic process, Woolley and colleagues could manage to harvest the first human collagenase out of rheumatoid synovium that had similar structure as the one in *Xenopus*. In 1986 cDNA cloning of human fibroblast revealed the first structural aspects of MMP 1. Although MMPs consist of

multiple domains, sequence homology to the first discovered collagenase (MMP 1) is the crucial necessity to be among the 24 human matrix metalloprotease genes (Murphy and Nagase 2008). Because MMP 23 is duplicated they currently list 23 different Metalloproteases, classified in the MEROPS database as M10 [2]. Beside their appearance in human and frog they can be found in *Drosophila melanogaster* (fruit fly), *Hydra vulagaris* (hydra), *Caenorhabditis elegans* (nematode), *Paracentrotus lividus* (sea urchin) and plants (Murphy and Nagase 2008).

2.1.3 Structure and Regulation

Based on their different domains MMPs are divided into eight structural subclasses, three membrane bound and five secreting types (Hagemann *et al.* 2012). The secreting ones are: Collagenase, Gelatinase, Stromelysins, Matrilysins and others (Murphy and Nagase 2008). Within the group of Metalloproteases they are assigned to a special super family called Metzincin (Tallant *et al.* 2010). MMPs are composed of a Hemopexin (Hpx) domain out of 200 amino acids, a Pro peptide of around 80 amino acids, a variable Linker peptide and the catalytic Metalloprotease domain consisting about 170 amino acids (Nagase *et al.* 2006).

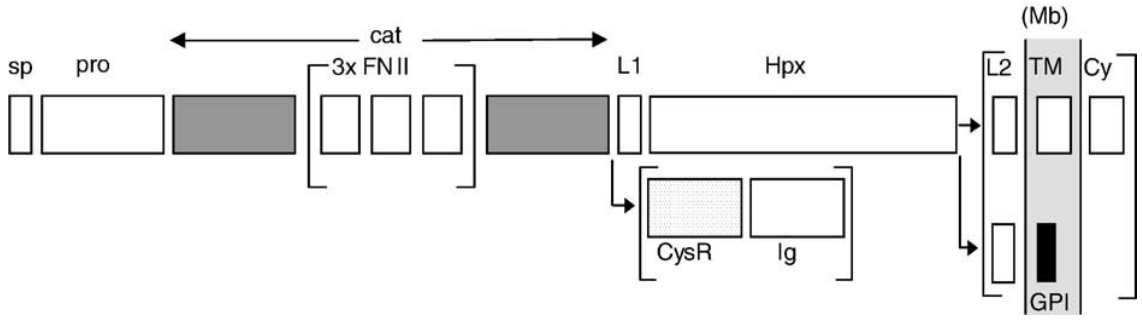

Figure 7. MMP structure: Sp: Signal sequence, Pro: Pro domain, Cat: Catalytic domain, L1: Linker 1, Hpx: Hemopexin domain, Mb: Plasma membrane (Nagase *et al.* 2006)

The zinc binding motif HEXXHXXGXXH of the metalloprotease domain and the cysteine switch motif PRCGXPD of the pro domain, coordinate MMP inactivation and, with an additional Met turn of the catalytic domain, define the collective structural aspects within this group. Both Met-turn and Zn^{++} binding motifs, are also present in the closely related A Disintegrin and Metalloproteases (ADAMs) family as well as in ADAMs with Thrombospondin Motifs (ADAMTS) (Tallant *et al.* 2010, Murphy and Nagase 2008, Nagase *et al.* 2006).

Most MMPs are produced as pre-pro enzymes. After stimuli by inflammatory phagocytes, connective tissue cells and other cells they are usually secreted into the extracellular space, although some, like MMP 9, can also remain inside the cell (Pasternak and Aspenberg, 2009).

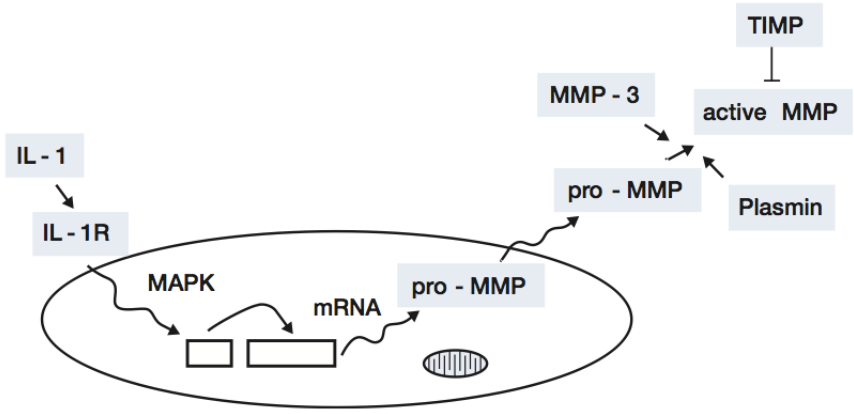

Figure 8. MMP activation (Pasternak and Aspenberg, 2009)

After cutting off the signal peptide during translation they become pro-MMPs, the inactive secreted zymogen. Proteolytic cleavage of the zinc - cystein alliance (shown for MMP3 in Figure 8.) activates the pro-MMPs, by exposure of their catalytic site. Different enzymes, reactive oxygen, denaturants or mercurial compounds and also the presence of other MMPs play a role in this initial activation (Pasternak and Aspenberg, 2009).

The conjunction of Metalloproteases activating other MMPs is displayed by the similarity of their expression patterns (combined expression of MMP 1 and MMP 10 as well as, MMP 2/14, MMP 24/2, MMP 25/2). Activated MMPs are affected by various endogenous inhibitors, like Tissue Inhibitor of Metalloproteases (TIMP), Reversion-inducing Cysteine-rich Protein with Kazal Motifs (RECK), Thrombospondin 2 or Alpha 2-macroglobulin. Last of which, facilitates an irreversible inhibition, by forming a complex with the enzyme. TIMPs on the other hand perform a reversible repression of MMP function and are the best understood MMP inhibitors. They consist of four proteins (TIMP 1 - 4). TIMPs 1, 2 and 4 are soluble and exist in most tissues, whereas TIMP 3 is connected to the extracellular matrix. Their complex formation is of non covalent nature and is either important for inhibition, as well as for activation of MMPs (Hagemann *et al.* 2012).

3. Extracellular Matrix

The Extracellular Matrix (ECM) is an omnipresent feature in all tissues and organs representing the non cellular component. From a general survey it is composed of water, polysaccharides and proteins. More accurately it can be divided into Proteoglycanes (PGs) and a non Proteoglycanes (Collagen, Fibronectin and Elastin), which represent the two major macromolecules of the ECM. Not only does the Extracellular Matrix function as a scaffold for the surrounding cells, it also regulates cell differentiation, homeostasis and morphogenesis (Frantz *et al.* 2010).

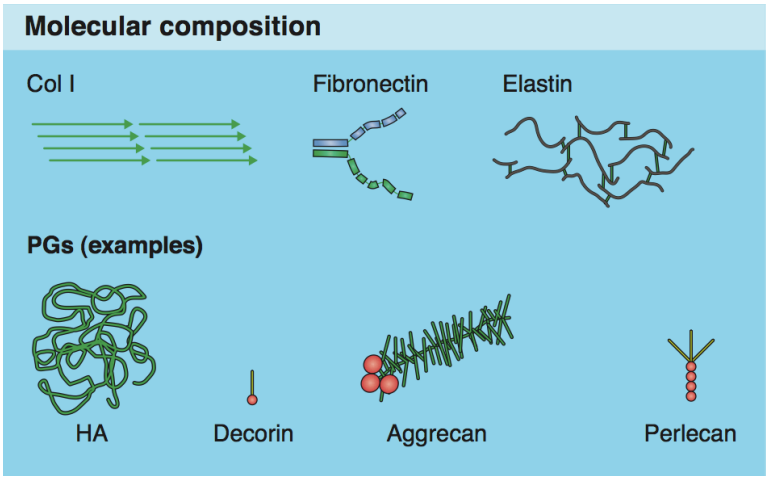

Figure 9. ECM molecular composition: Col I: Collagen, PGs: Proteoglycanes, HA: Hyaluronic Acid (Frantz *et al.* 2010)

Its unique composition for every tissue depends highly on the regulation between MMPs and TIMPs. A disturbance of this equilibrium can lead to fibrotic or degenerative processes (Figure 10.) (Pasternak and Aspenberg 2009).

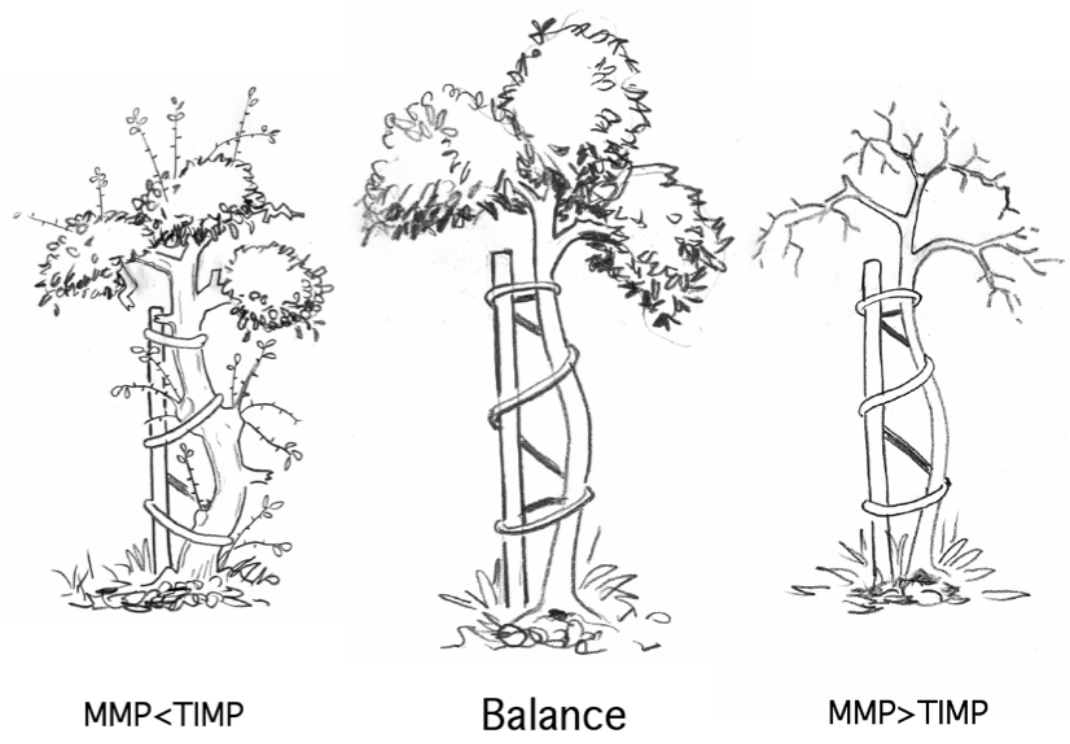

Figure 10. MMP / TIMP regulation describing fibrotic, normal, and degenerative equilibrium (Pasternak and Aspenberg 2009)

3.1 Extracellular Matrix and Matrix Metalloproteases in Cancer

Like every tissue, tumours are surrounded by the Extracellular Matrix. So far only four enzyme classes have been identified with the ability to break down the highly dynamic ECM: Matrix Metalloproteases, Aspartatic-, Cysteine- and Serine proteinases. By eliminating this structural barrier, cells of the primary tumour can invade, intravasate and metastasize to variable locations (Schröpfer *et al.* 2010).

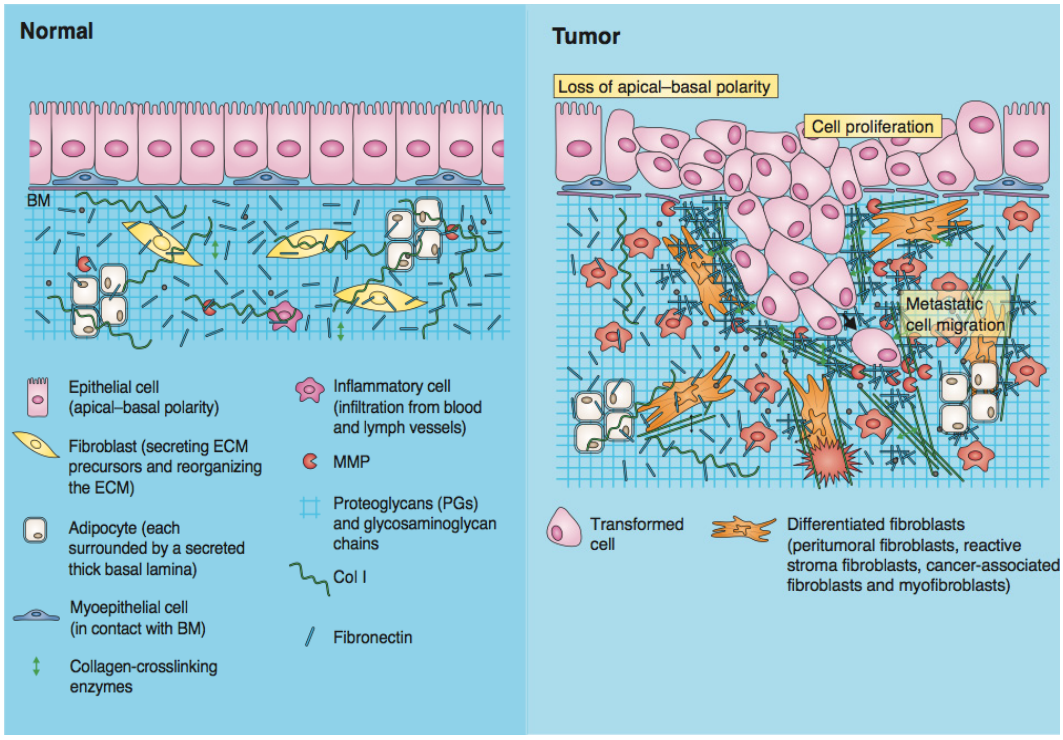

Figure 11. Physiological versus cancerous ECM (Frantz *et al.* 2010)

Metastasis is a process established in multiple steps. It requires very specific changes and interactions to overcome the physiological protection barriers of the human body. ECM remodelling can be considered the initial step of the Epithelial Mesenchymal Transition (EMT). The metastasis cascade explains how cells breach the Extracellular Matrix and disconnect from the primary tumour to start their migration and invasion of ambient tissue. Degradation of the Extracellular Matrix is the main key for tumour malignancy. After entering vessels and circulating throughout the body, the cells extravasate at various locations and form

secondary metastatic sites. MMPs have been investigated to be critical molecules that tumour cells recruit for this purpose (Deryugina and Quigley 2006). A study from Liotta and colleagues showed that the ability of tumours to degrade the ECM, correlated directly with their metastatic potential and could be linked to high MMP expression (Liotta *et al.* 1980). On the other hand, studies with specific Matrix Metalloprotease knock down mice showed lower tumour progression and less invasion (Konstantinopoulos *et al.* 2008). Additional studies of Mira, Lacalle, Marques and Martinz showed that proteolytic ECM degradation altered the available amount of growth factors (part of the ECM) and enhanced tumour progression and invasion by paracrine stimulation (Mira *et al.* 1999). In 1994 Kohka and colleagues had already demonstrated the converse effect, that overexpression of MMP inhibitors (TIMPs) reduced tumour metastasis and growth (Kohka *et al.* 1994). From there, the thought of using MMPs as new possible therapeutic targets was born. These so called MMPIs (MMP inhibitors) were developed in large numbers and showed promising results in *ex vivo* studies. But clinical trials could not offer satisfying results and an increase in overall survival in tumour patients could not be shown (Konstantinopoulos *et al.* 2008). Though to a high toxicity of the agents clinical trials were aborted in phase I and II (Saftig & Reiss, 2011). Probably because Matrix Metalloproteases have not only effects on tumour growth, angiogenesis, immunity regulation and anti-apoptotic abilities (Overall and Kleinfeld 2006), but play important roles in physiological processes (tissue remodelling, embryonic development, morphogenesis) as well (Hagemann *et al.* 2012). The role of MMPs in cancer is not yet sufficiently illuminated. Konstantinopoulos showed that Matrix Metalloproteases do not necessarily have effects on tumour progression. Some even inhibit it, or play other roles, for example in angiogenesis, immunity or host defence mechanisms (Figure 12). Therefore it is highly important to differentiate between these diverse sorts of MMPs (Konstantinopoulos *et al.* 2008).

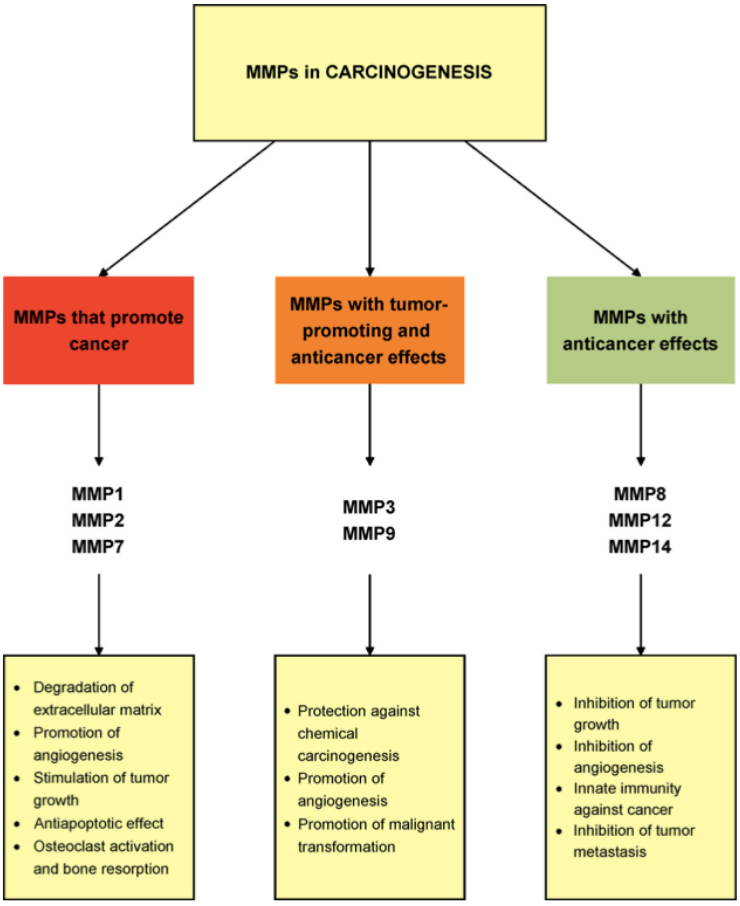

Figure 12. Different MMP functions in carcinogenesis, The schematic overview summons MMP types and functions. It explains that some MMPs either promote cancer while others inhibit it. Additionally there are some that show ambivalent behaviour (Konstantinopoulos *et al.* 2008).

4. A Disintegrin and Metalloproteases

4.1 Overview

The A Disintegrin and Metalloprotease family (ADAMs) is, like MMPs, a member of the Metzincin superfamily, a zink (Zn^{++}) dependent endopeptidase family consisting of 25 human genes, including one ADAM-like decysin gene (ADAMDEC1) and 4 pseudogenes (Shiomi *et al.* 2010).

4.2 History

In studies using guinea pig gametes, Primakoff investigated a drastic reduction of sperm-egg fusion (minus 75%) by applying an antibody blockage, with a protein he called PH-30, which was the foundation of the new protein class that would later be called ADAMs (Primakoff *et al.* 1987). Wolfsberg and colleagues figured out that this new family encoded proteins with a length between 750 – 800 amino acids, having a Pro-, Disintegrin-, Metalloprotease-, EGF-, Cysteine rich, cytoplasmatic and transmembrane domains that were commonly known from snake venom proteins. The relation to the soluble Snake Venom Metalloproteases (SVMPs) and their variable domains, with their unique constellation of cell surface proteins, created the name ADAMs (Wolfsberg *et al.* 1995). The presence of the Metalloprotease domain, the Disintegrin and the Cystein-rich domain is the reason why they are sometimes equally referred to as MDC family. Their expression could be found in *Xenopus, Drosophila, Caenorhabditis elegans* and human, but showed no tracks in plants, *Escherica coli or Saccharomyces cerevisiae* (Seals and Courtneidge 2003).

,

4.3 Structure

Their ability to cleave proteins through hydrolysis counts ADAMs into the group of proteases (peptidases) (Gomis-Rüth 2003). Facilitated by an active metal ion, furthermore characterizes them as Metalloproteases (Ramussen and McCann, 1997) and because of the zinc atom and the Met-turn, they get categorized into the supergroup of Metzincins (Gomis-Rüth 2003). The Metalloprotease domain is considered to be their active centre and consists of an enzymatically active ion and a methionine that is conserved in the Met-turn motif, plus a highly conservative amino acid sequence (HEXXHXXGXXH) coordinated by three histidins. These three and methionine control the catalytic function of ADAMs (Seals and Courtneidge 2003).

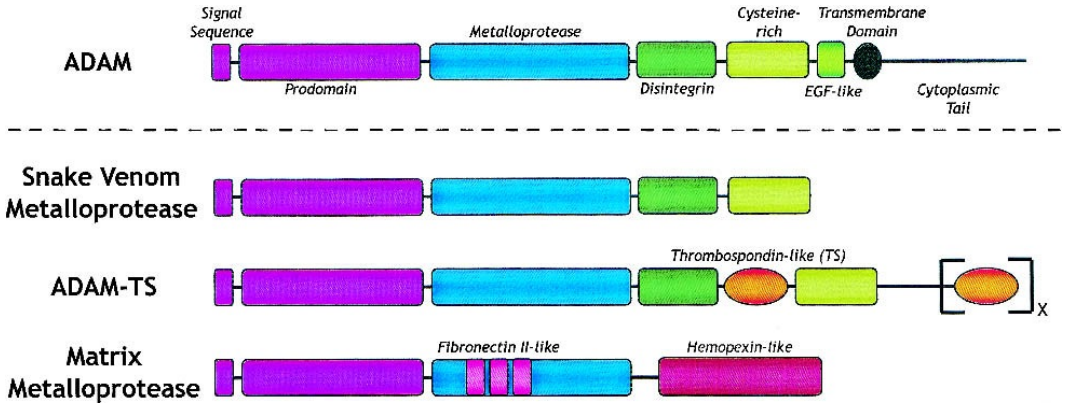

Figure 13. Domains of different Metalloproteases (Seals and Courtneidge 2003)

4.4 Functions

Degrading Extracellular Matrix components is often thought to be exclusively the work of MMPs. But also ADAM 10, 13 and 15 have shown to cleave certain ECM proteins. Their main function though, lies in cutting off membrane bound peptides,

through shedding, which is regulated by many different stimuli (diverse intracellular Ca^{++} concentration, growth factors, or osmotic changes, as well as non identified serum factors) (Seals and Courtneidge 2003). ADAMs are shown to regulate certain cell-cell interactions and play considerable roles in muscle development, fertilization, embryogenesis or neurogenesis (Gomis-Rüth 2003). ADAM 2 has a key role in the initiation of fertilization and ADAM 9, 19 and in particular ADAM 12 show regulating features in muscle development. ADAM 17 plays a crucial role in inflammation mechanisms. In cardiac hypertrophy, blocked ADAM 12 is found to beneficially support treatment. Data from studies with bacterial lung infections indicated that anti-sense ADAM 10 alters clinical outcome in cystic fibrosis patients. In the multifactorial etiology of asthma and bronchial hyper responsiveness, polymorphisms found on chromosome 20 suspect ADAM 33 to be involved in its occurrence (Seals and Courtneidge 2003). Many diverse enzymes are responsible for ADAM activation, such as specific inhibitors of Metalloproteases (TIMPs) (Bret *et al.* 2011). Regulating their activation might therefore be a considerable approach in anti-cancer treatment.

4.5 ADAMs in Cancer

Tumour cells can provide themselves with growth factors through shedding by autocrine or paracrine release, which contributes to their growth and metastasis potential (Mochizuki and Okada 2007). Current studies point out that expression mismatches in ADAMs are directly linked to poor clinical outcome and overall survival rate (for example ADAM 10 in osteosarcoma) (Zhao et al. 2014). Overexpressed ADAM 12 and 17 have been found in small cell lung cancer (Shao *et al.* 2014, Kamarajan *et al.* 2013). Similar findings have been postulated for ADAM 10 in neuroblastoma and pheochromocytoma (Yavari et al. 1998). Their involvement in physiological cell migration (Seals and Courtneidge 2003) raised the question if they are also present in tumour migration.

4.6 MMPs and ADAMs Combined Functions

ADAMs and MMPs have many diverse effects that regulate physiological as well as pathophysiological cascades. Their activity can be described in almost every somatic cell, operating with direct effects, or facilitating effects at a cellular level. Figure 14. shows a summary of Metalloprotease functions (Shiomi *et al.* 2010).

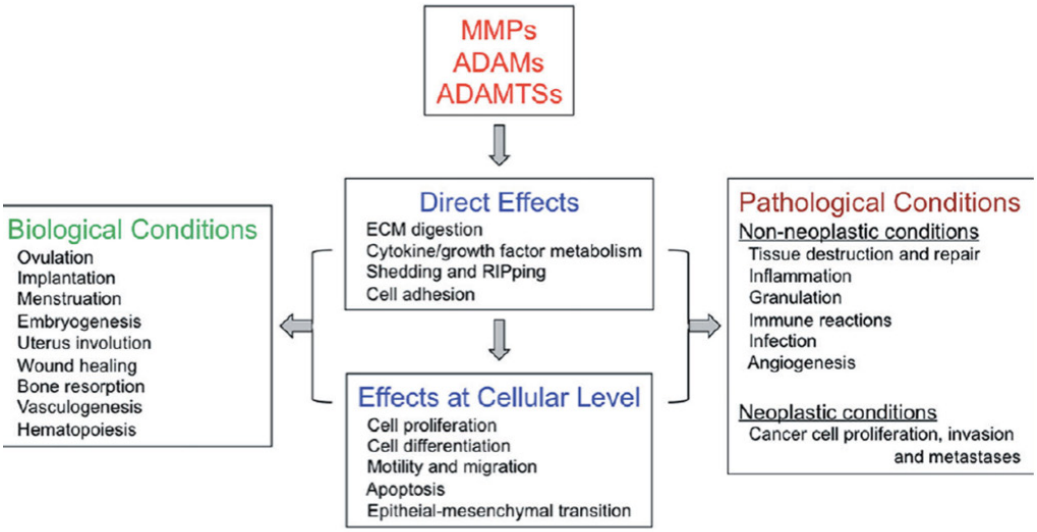

Figure 14. Combined effects of MMPs and ADAMs (Shiomi *et al.* 2010)

5. Expression Profiles

Many different studies suggest that there is a close relation between Metalloprotease expression and metastasis (for example in thyroid, breast, gastric, rectal or lung cancer) (Velinov *et al.* 2010). As described by Hida and Hamada in different non-small-cell lung cancers (adenocarcinoma / squamous cell carcinoma), protease expression can vary even in one and the same organ (Hida and Hamada 2012). Gene profile analyses have the ability to show proteases expression patterns for a certain tumour cell line (respective tumour tissue). Because of the different MP functions in carcinogenesis (Konstantinopoulos *et al.* 2008), evaluation of these patterns will help to optimize strategies for clinical trials, which currently still lack satisfying outcome (Saftig and Reiss, 2010), and to design accurate biomarkers for early tumour detection or trend estimation. Though to a current lack of these profiles for most Neuroendocrine Tumour cell lines, it was our goal to create valid expression patterns for numerous Medullary Thyroid Carcinoma cell lines (MTC) and Small Intestine NETs (SI-NETs) cell lines. Our collective contains 2 SI-NET lines (KRJ-I, P-STS) and 10 MTC (BOJO, GRS-IV, GRS-V, HEVE-II, MTC-SK, OEE-III, RARE, SHER-1, SINJ, TT) cell lines.

6. Aim of this Work

Analysis of tumours and their protease expression has already contributed to a more profound understanding of metastatic behaviour, which forms the basis to adequate anti-cancer therapies and the development of specific diagnostic tools. Previous studies suggested that there is a close relation between Matrix Metalloprotease expression and metastasis in thyroid-, breast-, gastric-, gynecological-, rectal-, lung cancer and glioblastoma (Velinov *et al.* 2010, Hagemann *et al.* 2010, 2012, Schröpfer *et al.* 2010) Also ADAM overexpression could be directly linked to poor clinical outcome and overall survival rate in cancer patients (ADAM 17 / non small lung cancer and ADAM 10 / osteosarcoma) (Ni et al. 2013, Zhao et al. 2014). Furthermore Hida and Hamada described that

neoplasms from the same anatomic origin can vary strongly in their metalloprotease expression (Hida and Hamada 2012). For a better understanding of tumour behaviour some colleagues have already provided protease expression profiles for various cancer types. (Hagemann *et al.* 2010, 2012, Schröpfer *et al.* 2010).

Until now, however, a systematic expression analysis of MMPs and ADAMs does not exist for Neuroendocrine Tumours. Our hypothesis therefore is that like in other tumours, MMPs and ADAMs do have an impact on growth and development of Neuroendocrine Tumours. Hence in the first attempt, a systematic analysis is of particular importance. The specific aim of this work was to explore the expression of MMPs and ADAMs in 12 different Neuroendocrine Tumour cell lines.

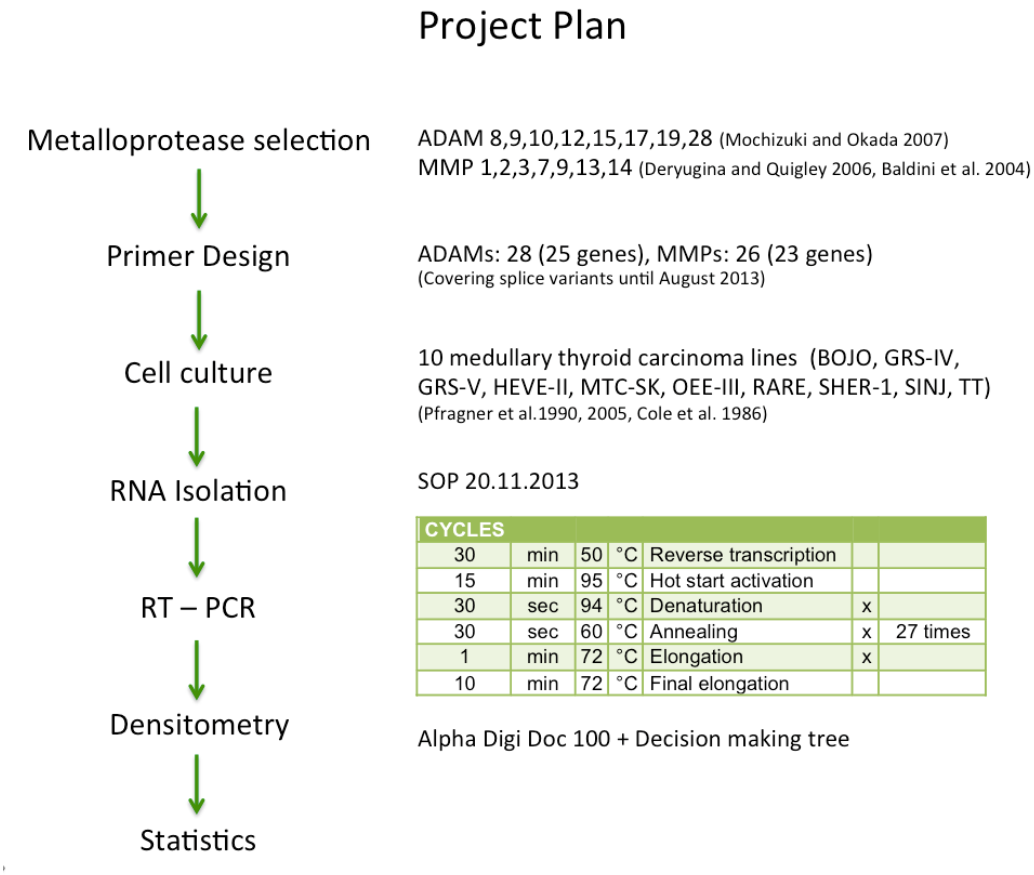

Figure 15. Project Plan

Material and Methods

7. Cell Culture

7.1 Introduction

During 1907 - 1910 an anatomy professor, named Ross Granville Harrsion, established a way to grow cells in culture. Based on the works of Ringer and others, he finished what would be referred as one of the ten greatest discoveries in medicine. John F. Enders was one of the first that showed the impact of this new method by growing poliomyelitis virus in culture which later on lead to the development of a polio vaccine for which in 1954 he and his colleagues were awarded the Nobel Price (Schiff, 2002).

In this work we cultivated 12 different Neuroendocrine Tumour cell lines. 2 derived from the small intestine (SI-NETs: P-STS, KRJ-I), and 10 from the thyroid gland (BOJO, GRS-IV, GRS-V, HEVE-II, MTC-SK, OEE-III, RARE, SHER-1, SINJ, TT). All cell lines, expect TT, were established by Prof. Roswitha Pfragner at the Institute of Pathophysiology and Immunology of Graz. Only MTC-SK, SINJ and KRJ-I are currently available to the public and can be found at the European Collection of Cell Cultures (ECACC). The characteristics of the 10 Medullary Thyroid Carcinoma and 2 SI-NET cell lines have been summoned in Table 1.

8. Cell Lines

8.1 Medullary Thyroid Carcinoma Cell Lines

MTCs derive from the parafollicular C-cells. They are calcitonin producing tumours that stand for approximately 5 -10% of thyroid neoplasms. They can occur separately or be a part of Multiple Endocrine Neoplasia (MEN2A, MEN2B). Currently surgical removal is the only curative option but with poor prognosis (Pfragner *et al.* 2005). The cell lines we used are summoned up in Table 1.

Table 1. Cultivated MTC cell lines (Pfragner *et al.* 1990, 1996, 2005)

MTC cell lines	Tumour stage	Sex/ age	RET status	Growth pattern	Immunocytochemical characterisation (CT, CGRP, GRP, NSE)			
BOJO	pT4N2M1 lymph node	M- 69	-	suspension	+	+	+	+
GRS IV	pT4N1M1 lymph node	M-55	-	suspension	+	+	+	+
GRS V	pT4N1M1 lymph node	M- 55	-	suspension	+	+	+	+
HEVE-II	pTxN1bMX	F/ 44	-	suspension	+	+	+	+
MTC-SK	pT4N1MX lymph node	F/ 51	-	suspension	+	+	+	+
OEE-III	pT2aN1bM1 lymph node	F/ 53	M918T	adherent	+	n.d.	n.d.	+
RARE	pT4N2MX lymph node	F-53	-	suspension	+	+	+	+
SHER-I	pT4N1MX primary tumour	M-72	-	suspension	+	n.d.	+	+
SINJ	pT2N1M0 lymph node	M-28	-	suspension	+	+	+	+
TT	- Lymph node	F/ 77	M634T	adherent	+	+	+	+
					(Cote *et al.* 1986, Zabel and Grzeszkowiak, 1997)			

8.2 Small Intestine Neuroendocrine Tumour Cell Lines

The establishment especially of neuroendocrine cell lines is a very difficult process, due to their low mitotic rate and small amount of tissue samples. Small Intestine NETs (SI-NETs) derive from the enterochromaffine cells (Pfragner *et al.* 2009). The 2 well-established and characterised cell lines (P-STS and KRJ-I) have been additionally used for our investigations.

Table 2. Cultivated SI-NET cell lines (Pfragner et al. 2009)

SI-NET cell lines	Tumour stage	Sex / age	Growth pattern	Immunocytochemical characterisation
KRJ-I	- primary tumour	M-75	suspension	Serotonin+, Chromogranine+ Somatostatin-, Gastrin-, Glucagon-, Pancreatic Polypeptide-;
P-STS	pT4pNpos(9/15)nM1 primary tumour	M-42	adherent	Chromogranin A +, HISL 19+, NSE +, Insulin -, Glucagon-, Pancreatic Polypeptide-, Gastrin-, Serotonin-, Somatostatin-alpha-HCH-;

9. Cell Culture Aspects

All cell lines were primarily defrosted, cultivated and harvested for experimental uses, or put into liquid nitrogen tanks (-196°C) for a later purpose.

Cultivation of the cells was obtained in a sterile lab class 2 under guideline approved circumstances. Disinfection (EtOH 75%) of hands, laminar flow, work place and all used chemicals and materials in contact with the tumour cells, as well as separate laboratory clothing and shoes were obligatory. We could not detect any contamination in the above mentioned cell lines during our trials, even though no antibiotics had to be used. The cell culture medium was assembled as suggested by the American Type Culture Collection (ATCC).

The principle of cell culture is to create an environment similar to the body, where cells can grow under relatively constant conditions. To provide this, it is of high importance to supply the cells with the right amount of nutrients, an optimum quantity of liquid, containing the right osmolality (300 mosmol/kg H_2O), pH (7,2 – 7,4) temperature (37°C) and CO_2 load (5%). While cells grow and divide themselves, the create by-products which need to be eliminated. Though to the fact that there are also growth factors, hormones and other metabolites produced during cell division, a well thought through protocol, when and how much to remove and to supply, is necessary for a strong cell viability. This makes cell culture a highly interesting and very challenging method, in which sophisticated trials close to *in vivo* conditions can be examined (Rinner 2013, ed. Alberts *et al.* 2011).

9.1 Medium Change / Splitting

Based on growth dependency, cells were either fed with medium or splitted into new charges. Most cell lines had constant splitting and feeding protocols (mostly well established lines). But nevertheless minor variations appeared throughout the charges and were managed individually to keep a standardized cell count. Before

a feeding/splitting process, all cells were examined under the microscope, to check for contamination, appearance and numbers. According to their growth pattern, old medium was removed (completely or partially) and fresh medium was added. (To see which cell line was treated with which medium please observe Table 3.) There were slightly different protocols for adherent and soluble cell lines. Suspension cell lines could be harvested easily, whether cell detachment techniques had to be performed for adherent cultures (mentioned below). 37°C with 5% CO_2 was considered as the standard incubation condition for all cell lines. If cell density reached a certain limit (usually around 90% density) we splitted the population and created an independent new charge. Splitting was more complicated for adherent cell lines, because these cells had to loosen their grip to the surface. This could be achieved mechanically with a cell scraper, or chemically *via* accutase, trypsin, chymotrypsin or EDTA, depending on the experience with these agents on the used cell line. The mechanism of these enzymes is to bind different ions, that are important for the interaction of extracellular matrix compounds, and to derange the cell to surface binding. To prevent cell damage agents were applied no longer than necessary. To create a working environment for these proteases, cells had to be washed clean from different substances, like Fetal Bovine Serum (FBS), that could interact with their function. Under the laminar flow bench we applied 10ml (preheated at 37°C) of Hanks Balanced Salt Solution (HBSS) buffer, mixing it with the old medium. We let the buffer react for 30 seconds and then removed it. Followed by 5ml of enzyme and after incubation for 10 minutes, we controlled cell dissolution by microscopic evaluation and gently tapped the flask bottom for a full dissolution if necessary. After adding new medium (preheated at 37°C) enzyme function was blocked. After strirring the cells by pipetting a couple times with a 1000ml tip, we centrifuged them until they formed a pellet. The overlap was thrown away and the pelleted cells were resuspendet in fresh medium. After a last microscopic control the cells were put back into the incubator (37°C, 5% CO_2) (Rinner 2013, ed. Alberts *et al.* 2011).

9.2 Thawing and Freezing

Unused cells were kept at -196°C in liquid nitrogen. This method of conservation is called kryo-conservation. For a continuous cell line this offers many positive aspects like reduced costs and low work effort. Additionally, conservation in liquid nitrogen is an excellent protection from contamination and genomic variability. To survive this freezing process cells get treated with antifreeze substances like glycerine or dimethylsulfoxid (DSMO). In the process of thawing these substances have a toxic potential, which is why it is recommended to thaw cells under 1 minute. Because of the possibility to dilute the toxic compounds more rapidly. Another point is the development of ice crystals inside the cells, a process called recrystallization, which happens to take place at about -60°C. This critic phase (-15°C to -60°C) is the major factor for cell survival in freezing and thawing processes. This is due to osmotic changes and direct ice crystal damage (Mazur 1984, Pegg 2002). For freezing, 1ml of pre-warmed medium was added to the cells and then the mixture was put in a 2ml cryo tube. After that, 100µl antifreeze substance (DSMO) was added and the tube was stored overnight at -80°C before put in liquid nitrogen (Rinner 2013).

9.3 Culture Medium

Table 3. Cell line cultivation

MTC and SI-NET culture mediums	
P-STS	Hams F12 + 10% FBS
KRJ-I	Hams F12 + 10% FBS
BOJO	Hams F12 + 10% FBS
GRS-IV	Hams F12 + 10% FBS + L-Glutamine
GRS-V	Hams F12 + 10% FBS + L-Glutamine
HEVE-II	Hams F12 + 10% FBS
MTC-SK	Hams F12 + 10% FBS
OEE-III	M199 + Hams F12 (50:50)
RARE	Hams F12 + 10% FBS
SHER-I	M199 + Hams F12 (50:50)
SINJ	Hams F12 + 10% FBS
TT	(Ref. Cote *et al.* 1986)

9.4 Cell Culture Materials

Table 4. Cultivation instruments

INSTRUMENTS AND MATERIALS	MANUFACTURER
Nitrogen tank	Chart Industries
Digital camera system (DS-L1)	Nikon
Fridge -20°C / +4°C	Liebherr
Freezer – 86°C	Heraeus
Incubator HERAcell 240	Heraeus Instruments
Inversion microscope (Eclipse TE 300)	Nikon
Pipettes (Pipetman P10, P20, P200, P1000)	Gilson
Mikroscope (BX40F3)	Olympus
Scale (SBC 21)	Scaltec Instruments
Mixer (Ikamag Ret)	Ika
Luminometer mediators PhL	Mediators Diagnostika

Centrifuge (GS 6)	Beckmann
Sterilisator(ST640)	Heraeus Instruments
Laminar flow work place (HERA Safe)	Heraeus Instruments
Wather bath (DC5)	Haake
Glas bin	Schott
Cryo tubes 2ml	Carl Roth GmbH (E292.1)
Cell culture flask 75cm²	Sarstedt (83.1813.002)
QuadriPerm (Heraeus)	Sigma-Aldrich (Z376760)
Myco Alert	Lonza (LT07-218)
Object plate 76x26 mm	Carl Roth GmbH (H868)
Parafilm M	America National Can
Petri dish	
Tweezer	
Pipette laces 200µl	Carl Roth GmbH (B007.1)
Serological pipette laces 1ml	Costar (4011)
5ml	Sarstedt (86.1253.001)
10ml	Sarstedt (86.1254.001)
25ml	Sarstedt (86.1685.001)
Conical tubes 15ml	Falcon (352097)
50ml	Falcon (352098)
Pillar	NeoLab (2-1969)
Syringe filter tips PVDF 0,22µm	Carl Roth GmbH(P666.1)
96 well micro titer plate	Sarstedt (83.1835)

Table 5. Cell culture chemicals

CHEMICALS	MANUFACTURER
Accutase	PAA (L11-007)
Alpha D-Glucose Monohydrate	Carl Roth GmbH(67801)
HAMS F12 without L-Glutamin	PAA (E15-016)
DMSO	Sigma-Aldrich (D2650)
DMEM	GIBCO (22320)
Fetal Bovine Serum (FBS)	PAA (A11-151)
HEPES buffer 1M	PAA (S11-001)
MEM with Earle´s Salt without L-Glutamin	PAA (E15-024)
HBSS	GIBCO (14175)
L-Glutamin 200 mM	PAA (M11-004)
Sodium Pyruvate 100 mM	PAA (S11-003)
Non essential aminoacids	PAA (M11-003)
Penicillin-Streptomycin (P/S)	GIBCO (15140-122)
RPMI 1640 without L-Glutamin	PAA (E15-039)

10. Ribonucleic Acid Isolation

Ribonucleic Acid (RNA) isolation is the process of harvesting single stranded RNA out of the cell. It is facilitated through chemicals and differences in weight and solubility (Alberts *et al.* 2011). Extracting RNA offers insights into cellular transcription and can therefore only describe the cellular activity at the moment The chemicals that we used contain harming substances. To prevent contamination, working with protection gloves (Nitril) and glasses under laminar airflow is obligatory. A disinfected work area is another mandatory standard for good RNA extraction.

RNA extraction is mainly facilitated in two steps. To get RNA out of the cells they have to be lysed. This process sets all cell components free and creates a mixture of many different molecules. To separate RNA from the rest, chemical and physical techniques have to be applied. Another limiting effect is contributed by the ribonuclease (RNase) activity, of all living cells, which reduces the amount of RNA. (ed. Alberts *et al.* 2011).

To isolate RNA we used the Standard Operating Procedure (SOP) (version 20.11. 2013) of the Institute of Pathophysiology and Immunology of Graz. It is a single step method facilitated with TriReagentRT provided by the Molecular Research Center company. TriReagentRT is a chemical that consists of two toxic substances: phenol and guanidinthiocyanat. Therefore working under a laminar airflow bench is obligatory. Guanidinthiocyanat is a denaturating agent that partially inhibits RNase and lysates the cells. Phenol on the other side works as an organic solvent for DNA and proteins. With sour pH conditions two phases can be generated. One in which the RNA is solved (watery phase) and one consisting of phenol in which proteins and DNA can be found (Kirby 1956, Chomczynski and Sacchi, 1987),[3,4].

After adding Bromchlorpropane and centrifugation, these phases get properly separated. After division, the watery phase is removed leaving the red organic Phenol phase at the bottom. The RNA phase is put into a new Eppendorf 1.5ml tube and is washed with Ethanol (EtOH 75%) or (according to our protocol) with 500µl isopropanol, to precipitate the RNA. To reduce various salts that could harm the RNA another washing step with EtOH 75% is done. After another centrifugation round, the RNA pellet is diluted in distilled water and sometimes Diethylpyrocarbonate (DEPC) to deactivate RNases is applied (Chomczynski and Sacchi, 1987),[4].

11. Nano Drop

To quantify the amount of RNA we used NanoDrop ND1000 (PEQLAB), a spectral analysis machine that could measure the concentration and purification of the probes. The machine was calibrated with 2µl of nuclease free water (NFH_2O). Each probe (2µl) was applied for 3 times, after vortexing the samples to assure an equal RNA mix of the probes. After measurement, the probes were diluted to 100 ng/µl and stored at – 20°C [5]. To check RNA quality we performed gel electrophoresis.

12. Gel Electrophoresis

Gel Electrophoresis is an excellent method to identify, purify and separate DNA or RNA. By using a fluorescent intercalating substance called Ethidium Bromide (EtBr) that gets mixed into the Agarose gel. Then a current is applied which drags the negative charged nucleic acids in one direction. Therefore separation and visualization of the variable sized RNA/DNA probes can be managed. After pipetting the nucleic acid directly over the gel chamber holes, it sinks autonomously in the preformed gel chamber. Before, it is mixed with a loading dye that enhances its density and shows RNA/DNA position on the gel while it is running. Separation by size comes *via* electrolysis of water at the electrodes. To prevent pH changes, the liquid in the electrophorese chamber contains a buffer, Tris Aceteate EDTA (TAE). Additionally a standard runs next to the probes to determine their size. Because the substances are very carcinogenic it is of high importance to use nitrile gloves and a laminar flow work place while the EtBr is applied. In all our experiments we used 1% Agarose gels (50ml TAE buffer with 0.5g agarose) and added 4µl EtBr. Our probes were diluted with 6x Loading Dye (see Table 8) for the above mentioned reasons. The probes ran for 45 min at 70 volts, 400mA and 80 watts and were then examined with a transluminator (ed. Alberts *et al.* 2011, [6]).

13. Reverse Transcriptase Polymerase Chain Reaction

After RNA Isolation of P-STS, KRJ-I, BOJO, GRS-IV, GRS-V, HEVE-II, MTC-SK, OEE-III, RARE, SHER-1, SINJ and TT, we performed Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) to identify Metalloprotease gene expression in our Neuroendocrine Tumour cell lines. With the Qiagen One Step PCR Kit, a combination of reverse transcriptase and regular PCR compounds (5x buffer, dNTP mix, NF H_2O, enzyme mix, 5´ and 3´primer), we were able to amplify RNA probes in one step. The kit firstly transformed a single stranded RNA into a RNA/DNA complex. In a second step the enzyme cut out RNA bases and

created double stranded DNA sample called cDNA. A necessary step as PCR only works with DNA [7].

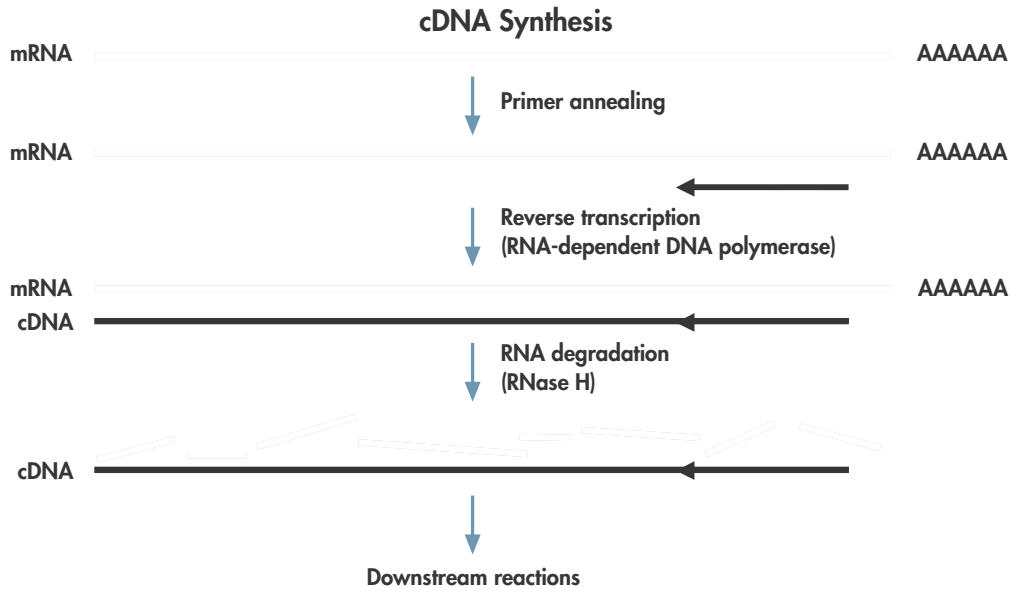

Figure 16. cDNA synthesis [7]

The RNA samples we used, were diluted with nuclease free water (NFH_2O) to a 100 ng/µl concentration and stored at -20°C. Self produced NFH_2O (17.07.09) was kept at 4°C. Primers and PCR kit were also stored at -20°C. All reagents except the enzyme were put on ice to slowly defreeze prior the experiment [7]. All used chemicals, instruments and materials are shown in Table 6.,7.,8.

Table 6. RT-PCR instruments

INSTRUMENTS	MANUFACTURER
Fridge ad freezer +4 und -20 °C	Liebherr
Electrophoresis chamber HE33	Hoefer Inc.
Pipetman P10	Gilson
P20	
P200	
P1000	
Microwave	Schaub Lorenz Sigma
Power Supply EPS 600	Power Supply EPS 600
Thermal Cycler C1000	Bio-Rad Laboratories
Thermomixer Comfort (1,5 ml)	Eppendorf
Minishaker MS 1	Ika
Mini centrifuge Rotalibo „Uni-fuge“	Sigma
Scale SBC 21	Scaltec Instruments
ChemiDoc XRS System	Bio-Rad Laboratories Hoefer Inc

Table 7. RT-PCR materials

MATERIAL	MANUFACTURER
PCR softstrips 0,2 ml	Biozym Scientific GmbH (71088)
Pipette filter tips (steril) 10 µl	Sorenson BioScience Inc. (10320)
200 µl	Sorenson BioScience Inc. (10350)

Table 8. RT-PCR chemicals

CHEMICALS	MANUFACTURER
DNA Standard 100 bp	Invitrogen (15628019)
Ethidium Bromide	Invitrogen (15585011)
6x Loading Dye	Fermentas (R0611)
LE Agarose	Biozym Scientific GmbH (840004)
OneStep RT-PCR Kit	Qiagen (210212)
TAE-Puffer 50x	self made

Before we could perform PCR we had to design primers (5´and 3´) that would express our wanted Metalloprotease genes. A detailed description of how these primers were designed can be found under the section (Primer Design). For our PCRs we used 27 cycles and stopped right during the exponential phase. Then we used Gel Electrophoresis to show protease gene expression in our tumour cell lines.

We provided a Mastermix (MM) for each PCR round, to shorten pipetting times and to reduce material wastage. For the positive control (L30) a separate MM had to be designed though to other PCR specifications.

Table 9. Mastermix composition

MASTERMIX	For all GENES and - CONTROL		+ CONTROL (L30)	
H_2O	11,6	µl	10	µl
5x RT buffer	4	µl	4	µl
dNTP mix (400µM/dNTP)	0,8	µl	0,8	µl
5´primer (0.6µM)	0,4	µl	1,2	µl
3´primer (0.6µM)	0,4	µl	1,2	µl
Enzyme mix	0,8	µl	0,8	µl
RNA (100ng/µl)	2	µl		
Total	20	µl	20	µl

Only H_2O, buffer, dNTPs and both primers were put in an Eppendorf tube together. The enzyme was applied right before the tubes were centrifuged and put into the PCR machine. After creating the MM for all our chosen genes, 2µl of RNA were pipetted in softstrips (PCR tubes) as described in Figure 17.

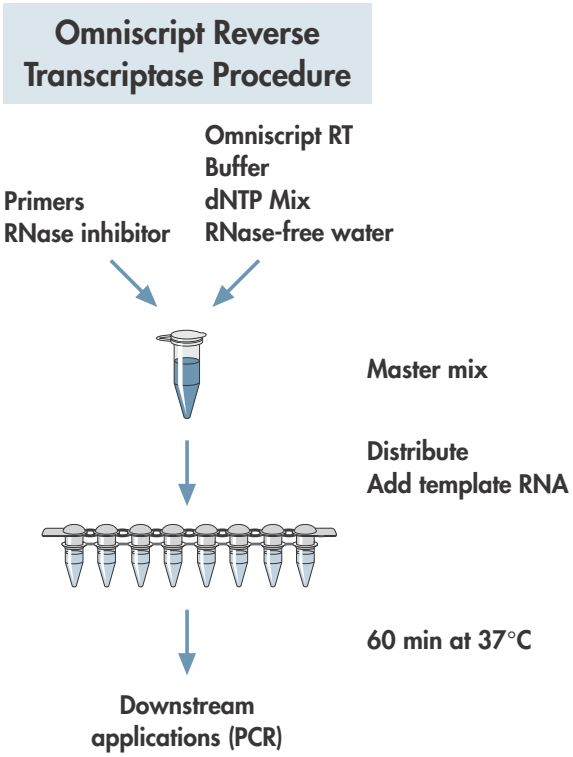

Figure 17. Reverse transcriptase procedure [7]

We performed RT-PCR, choosing 27 cycles to multiply our probes.

Table 10. Cycling protocol for RT-PCR

CYCLING PROTOCOL						
30	min	50	°C	Reverse transcription		
15	min	95	°C	Hot start activation		
30	sec	94	°C	Denaturation	x	
30	sec	60	°C	Annealing	x	27 times
1	min	72	°C	Elongation	x	
10	min	72	°C	Final elongation		

To evaluate gene expression we used Gel Electrophoresis and established the profiles [6].

14. Primer Design

UCSC Genome Browser [8] offered us essential information about each gene. Careful consideration must be given to ensure that the primers are specific for the gene of interest and that they only amplify cDNA and not the genomic DNA. In the cell, processing DNA to m-RNA requires a couple steps: first step, the splicing, may lead to various so called splice variants. There can be many different possibilities for enzymes involved in this process to cut out introns and sometimes even parts of coding RNA. Also noncoding sequence can be left within. This fascinating way generates many different proteins from only one template and changes the appearance of m-RNAs in size and information, when fully processed. In order to cover all these different variants, we used the *Ensembles* [9] graphic abstract to design overlapping primers that would cover all coding transcript variants (ed. Alberts *et al.* 2011).

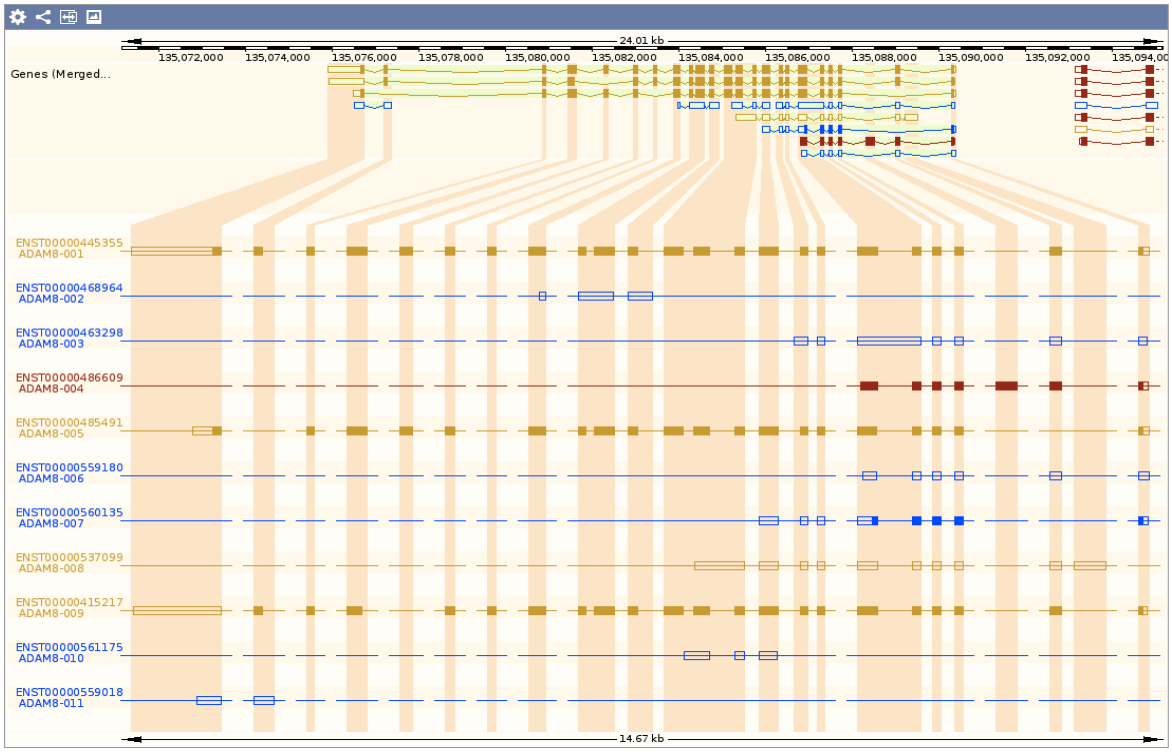

Figure 18. Splice variant overview in Ensemble [9]

Primers were designed with the programme Primer 3 [10]. As criteria, we configured to design primers to cover 2 exons, to distinguish between genomic DNA and experimentally generated cDNA - just in case the samples were contaminated with genomic DNA. We also set the PCR product size between 200-400 bp. In Silico Polymerase Chain Reaction (In Silico PCR) was performed to check selectivity and efficiency of the chosen pair and to show the potential genomic product size (which equals DNA). With the programme BLAT we could ensure, that our designed sequence could only be found on one specific DNA locus. Both tools were offered on UCSC Genome Browser. A short summary about gene information, location, product and genomic sizes as well as transcript information and both tests (BLAT, InSilico PCR) were created for each primer pair [8]. We generated 26 MMP primer pairs for 23 genes and 28 ADAM primers for 25 genes (Table 12 and 13).

Table 12. MMP primer pairs

MMP PRIMERs							
Ref Seq Nr.	**Primer**	**Exon**	**5´ FORWARD**	**Exon**	**3´ REVERSE**	**Product Size**	**Genomic Size**
NM_001145938	*hMMP1*	3	GGTCTCTGAGGGTCAAGCAG	5	AGTTCATGAGCTGCAACACG	207 bp	1487 bp
NM_001127891	*hMMP2*	2	ACCCAGATGTGGCCAACTAC	4	GAGTCCGTCCTTACCGTCAA	253 bp	2274bp
		8	CTGGAGCACTCCCAAGACC	9	CTGAGCGATGCCATCAAATA	348 bp	1411bp
NM_002422	*hMMP3*	2	CCGAGAAATGCAGAAGTTCC	3	CAACAGCAGAATCAACAGCA	212bp	304 bp
NM_002423	*hMMP7*	2	GAGTGCCAGATGTTGCAGAA	4	GTGAGCATCTCCTCCGAGAC	311 bp	2854 bp
	hMMP8	4	TTTGATGGACCCAATGGAAT	5	GTTGCTGGTTTCCCTGAAAG	222bp	3047bp
NM_004994	*hMMP9*	3	CATCGTCATCCAGTTTGGTG	6	GCCTTGGAAGATGAATGGAA	394 bp	1033 bp
NM_002425	*hMMP10*	2	ATTTTGGCCCTCTCTTCCAT	4	ATTTGTCCGCTGCAAAGAAG	230 bp	2411 bp
NM_005940	*hMMP11*	7	TACTGGGTGTACGACGGTGA	8	AGAAGTCAGGACCCACGAGA	349 bp	1276 bp
NM_002427	*hMMP13*	5	AGTTCGGCCACTCCTTAGGT	7	GGAAGTTCTGGCCAAAATGA	325 bp	3060bp
NM_004995	*hMMP14*	2	GCCATGCAGAAGTTTTACGG	4	CTTGGGGGTGTAATTCTGGA	204 bp	851 bp
NM_002428	*hMMP15*	4	GGCCGACATCATGGTACTCT	5	GTCAACGTCCTTCCACTGGT	280 bp	634 bp
NM_005941	*hMMP16*	9	ATCCCAAGCCAATCACAGTC	10	AGGAGGCATAAGGCCAAGAT	316 bp	5189 bp
NM_016155	*hMMP17*	3	CCACCAAGTGGAACAAGAGG	4	GGGTAGCCGTCGTTATGGT	208 bp	2039 bp
		8	CAGCGACCACAAGATCGTC	9	ATGTGCCTCGTGTGGTCAT	207 bp	4559 bp
		9	CTGTACTGGCGCTACGATGA	10	GGCTCTGGTCATGTTGTCCT	310 bp	1175 bp
NM_002429	*hMMP19*	1	GCTTCCTACTCCCCATGACA	3	GATCCTCTAGGCCACAACGA	243 bp	1664 bp
		7	TGCTTTCAAGGGGGACTATG	8	GCCAATAGAGAGCTGCATCC	245 bp	405 bp
NM_004771	*hMMP20*	4	CGACAATGCTGAGAAGTGGA	6	TCACAGCGTCAAAGGATGAG	302 bp	3368 bp
NM_147191	*hMMP21*	3	TTCACTTTGACGACGACGAG	5	CAGCCAGTGAGGATTTGGAT	385 bp	2262 bp
NM_006983	*hMMP23b*	1	CTGAGCCCCACAGCAAGT	2	GCTGGAGTCAGCGTGTAGC	274 bp	682 bp
		5	GCCTGATGCACTCACAACAC	7	TTTCCCTTTCTTGTGGAGGA	341 bp	519 bp
NM_006690	*hMMP24*	8	TGAAGGCATTGACACAGCTC	9	CGCTCAGTTTCTGGTTGTCA	242 bp	2678 bp
NM_022468	*hMMP25*	7	GATCGATGTGAGGGCAATTT	8	TAGGTCTTCCCGTTCTGTGG	344bp	989bp
NM_021801	*hMMP26*	3	GCAGAATGGAGATGCAGACA	4	TTGGATATCATCGGCACTGA	313 bp	1404 bp
NM_022122	*hMMP27*	5	CAGCCTTGATGTTCCCAAAT	7	GATGGCCAGAATGAAGCAAT	280 bp	1735 bp
NM_001032278	*hMMP28*	2	CAATGAACAGGTCCCCAAAG	3	AAAGCGTTTCTTACGCCTCA	232 bp	400 bp
		1	TTGAGACCTGGGACTCCTACA	2	CAGCCCGACCCATCTTTC	200 bp	510 bp

Table 13. ADAM primer pairs

ADAM PRIMER							
Ref Seq Nr.	**Primer**	**Exon**	**5´ FORWARD**	**Exon**	**3´ REVERSE**	**Product Size**	**Genomic Size**
NM_001278113	*hADAM 2*	12	TGGAAGCAGGAGAGGAGTGT	14	CCATTGATTCAGTCCACACG	272bp	2521bp
NM_003817.3	*hADAM 7*	12	TGCATGCTCAACATTCCATT	14	TTCCCCATGAAACAGTAGCC	344bp	2823bp
		4	ACCAGGCATCCTCAGATCAT	6	CCCCTGGAACAGTTTTTCTG	256bp	3017bp
NM_001109	*hADAM 8*	3	CACAACTTCACCCTCCACCT	6	AGGTGCTCAGCCTGGTACAC	316bp	834bp
NM_003816	*hADAM 9*	1	GTGTCCGGTGGTTGCTGT	3	TTCCTTTCCAAGTGAATAATATGCT	214bp	3832bp
NM_001110	*hADAM 10*	1	GGATCGATGTGCTGCTGTTA	4	TTCTCCTGGTGTGCACTCTG	1295bp	
		1	GGATCGATGTGCTGCTGTTA	2	CGCTGGTGTTTTTGGTGTAA	203bp	
NM_002390	*hADAM 11*	22	GTGGCTTCCTCCTCTGTGTC	24	GGCAGGGGGTTATGGATACT	355bp	536bp
NM_003474	*hADAM 12*	5	TCAGCACGTGTTCTGGTCTC	9	ACACTTCCACGCCTACCAAC	396bp	
NM_207197	*hADAM 15*	13	ATCTGACGGACCCTGTTGTC	15	AGGCTGGGTCCTACCTGTCT	385bp	596bp
NM_003183	*hADAM 17*	5	TGCAGTCTCCAAAAGTGTGTG	6	CCCTCTGCCCATGTATCTGT	194bp	1735bp
NM_014237	*hADAM 18*	5	GCATTGCCATTACCAAGGAT	6	TGTAGGGCTGGTCTTTCTGC	233bp	1200bp
		15	GTGCTCCATTTGCCTGTTTT	16	CATCAGCCACATAGGCATTG	264bp	2764bp
NM_033274	*hADAM 19*	15	AGGTGAATGTGGCAGGAGAC	17	CTTCTTCCCACAGCCTTCAG	344bp	2913bp
NM_003814	*hADAM 20*	1	AACAGGTTGGTCGTTTTTGC	1	CACATTCCTCCCCTTCTTCA	274bp	274bp
NM_003813	*hADAM 21*	1	ACTACTGTTTCTTCTGATGCACTG	2	GAACAACGTGTTTCTGGCCC	393bp	5478bp
NM_021723	*hADAM 22*	27	AAAAAGCCTGGAGATGGTGA	29	TTTGTTGCCTCCCAGGTTAC	291bp	3018bp
		3	GCCTTTGGAACGTCATTCAT	6	TGTGTGGTTCCCGTCATAGA	219bp	
NM_003812	*hADAM 23*	23	TATGAGCAGCTGTCCACTCG	24	CCTTGGGTCCTTCATCCTTG	164bp	1309bp
		1	CCACTCGATTCCAAGGGTAA	3	CATCCTTGTCACCCCAACTT	345bp	
NM_014265	*hADAM 28*	10	CTGGAACGACTGTGGGTCTT	11	GCTCAGTGCTTTGTCCATCA	200bp	3549bp
		18	GACCATGAGCTCCAGTGTCA	20	GGGTTGAACAGCCTTTACCA	255bp	6937bp
NM_001278127	*hADAM 29*	2	ATTCTCCCTGCAGTCTCACG	3	GGTCCAGAGCAGAAGTCAGG	300bp	44471bp
		1	CTAACCAAATGGGGTGGTGT	2	AGAAGTCAGGGCTGGAGTCA	215bp	
NM_021794	*hADAM 30*	1	ACCTTGCAGAGTACTGCGAC	1	TGCCCCAGCACATGAGATTT	372bp	372bp
NM_145004	*hADAM 32*	2	TCGTAATTCCAGAGAAAATCCAA	5	GTGTGACCATGGAATCTGGA	235bp	31722bp
		1	GAGAACGCTGTCCCATGAAC	4	GTGTGACCATGGAATCTGGA	366bp	43694bp
NM_025220	*hADAM 33*	4	CTGGCCCCAGGATACATAGA	6	GTTCCTTTCCAGGTGAGCAG	272bp	528bp

15. Densitometry

The software Alpha Digi Doc 100 served as a quantification system for our PCR results. By measuring the intensity of the probes through pixel accumulation and comparing them against the background of the Gel Electrophoresis picture, we quantified the amount of PCR products and provided a graphical overview of Metalloproteases expression in the different cell lines [11]. To do so we had to manually pick every single band in the gel by a defined frame and also set one as background (base value for exposure). After developing an analysis scheme, we determined the following standards to interpret our findings. Everything 2,4 times the intensity of our background (BG) was awarded with +++ expression, everything >1,5 more intensive as BG with ++ and all data over 1 was given a +. The background was defined as 1. If bands could be seen and the intensity was below 1 we awarded (+) for very low expression. Also bands had to be seen by eye to avoid false interpretation. Corrections have been made supported by the “Decision Making Tree” (see Figure 19.).

Table 14. Densitometry interpretation

Expression intensity	
> 2,4xBG	+++
> 1,5xBG	++
> 1x BG	+
< 1xBG	0
BG = Background = 1	

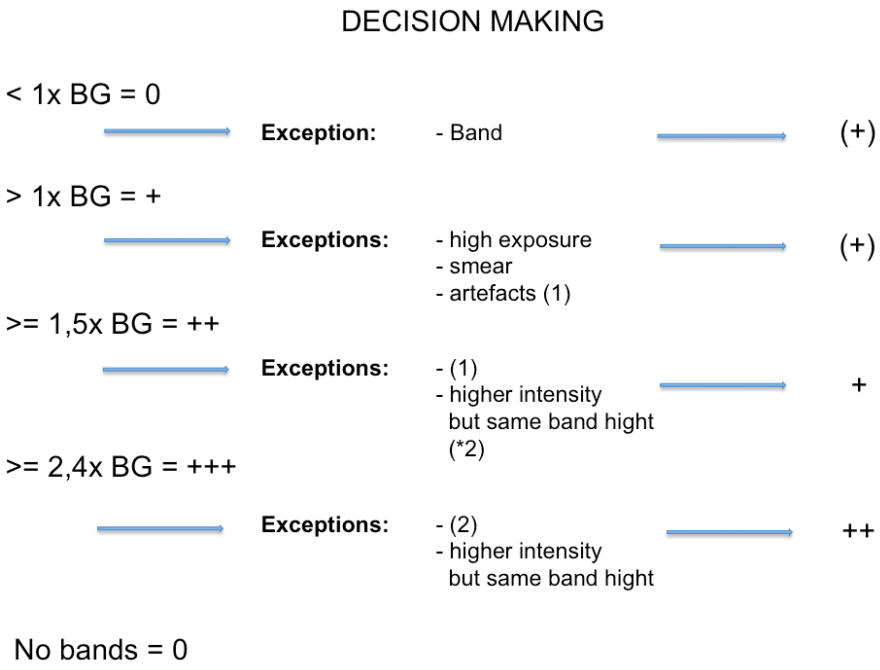

Figure 19. Decision Making Tree for densitometry results

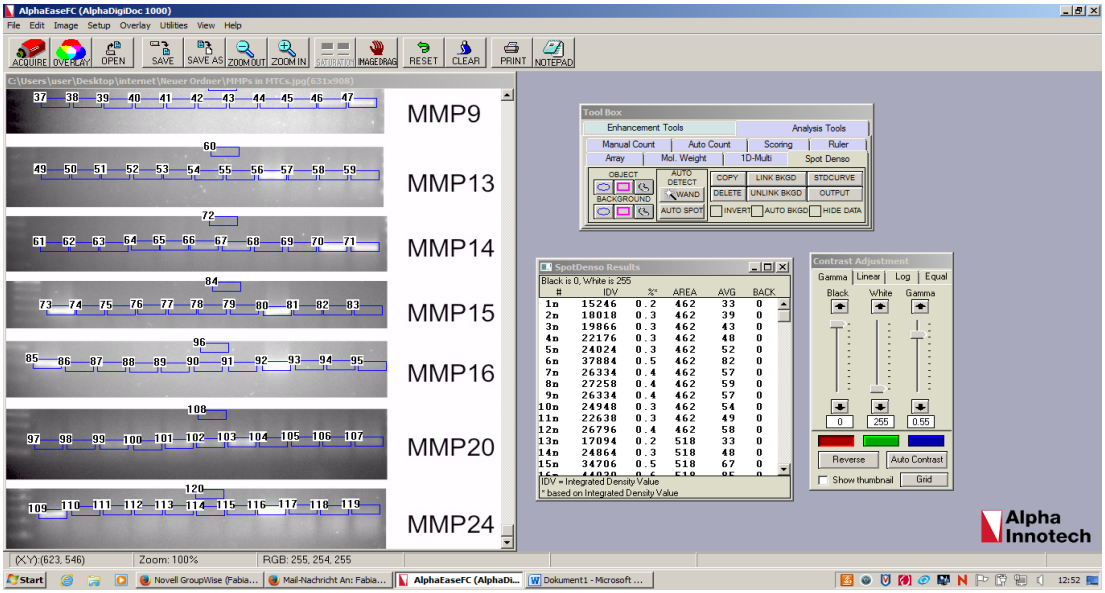

Figure 20. Alpha Digi Doc 100 program overview

							ERGEBNISSE
	Black is 0, White is						
	#	IDV	%*	AREA	AVG	BACK	
MMP15	1n	83628	25.9	828	101	0	2,29545455
ADAM17	2n	120888	37.4	828	146	0	3,31818182
ADAM18a	3n	33120	10.3	828	40	0	0,90909091
ADAM18b	4n	28152	8.7	828	34	0	0,77272727
ADAM22a	5n	20700	6.4	828	25	0	0,56818182
Background	6n	36432	11.3	828	44	0	1
	Black is 0, White is						
	#	IDV	%*	AREA	AVG	BACK	
ADAM22b	1n	104632	21.7	902	116	0	1,96610169
ADAM28a	2n	47806	9.9	902	53	0	0,89830508
ADAM28b	3n	48708	10.1	902	54	0	0,91525424
ADAM33	4n	44198	9.2	902	49	0	0,83050847
L30	5n	183106	38.0	902	203	0	3,44067797
Background	6n	53218	11.0	902	59	0	1
	Black is 0, White is						
	#	IDV	%*	AREA	AVG	BACK	
MMP1	1n	55965	11.9	861	65	0	0,66326531
MMP7	2n	78351	16.6	861	91	0	0,92857143
MMP13	3n	85239	18.1	861	99	0	1,01020408
MMP16	4n	114513	24.3	861	133	0	1,35714286
MMP20	5n	53382	11.3	861	62	0	0,63265306
Background	6n	84378	17.9	861	98	0	1
	Black is 0, White is						

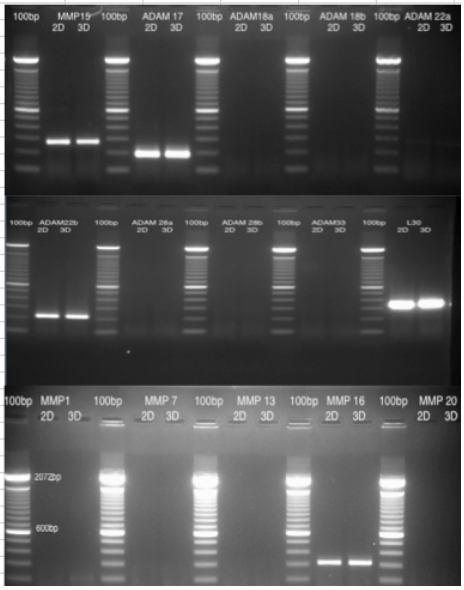

Figure 21. Densitometry results processed with Excel for Mac 2010

RESULTS

16. Cell Line Characteristics

To prove the content of neuroendocrine features in our cell lines, several standard Neuroendocrine Markers have been examined *via* RT PCR (listed in Table 15). The results show that not all NET cell lines express the whole range of markers used in this study (Figure 22 and 23).

Table 15. NET Markers used in this study [13]

Neuroendocrine Markers (human)	
L30	Ribosomal protein L30
ASCL1	Achaete-scute homolog 1
CUX1	Cut-like homeobox 1
CHGA	Chromogranin A (parathyroid secretory protein 1)
CHGB	Chromogranin B (secretogranin 1)
SCG2	Secretogranin II
SCG3	Secretogranin III
SCG5	Secretogranin V
SYPb	Synaptophysin b
M2BP	Lectin, galactoside-binding, soluble, 3 binding protein
ENO1	Enolase 1 (alpha)
ENO2	Enolase 2 (gamma, neuronal)

No.	Cell line	Tumour stage	Sex/ age	RET status	Growth pattern	Immunocytochemical characterisation (CT, CGRP, GRP, NSE)			
1	TT*	- Lymph node	F/ 77	M634T	adherent	+	+	+	+
2	BOJO	pT4N2M1 lymph node	M- 69	-	suspension	+	+	+	+
3	GRS IV	pT4N1M1 lymph node	M-55	-	suspension	+	+	+	+
4	GRS V	pT4N1M1 lymph node	M- 55	-	suspension	+	+	+	+
5	RARE	pT4N2MX lymph node	F-53	-	suspension	+	+	+	+
6	SHER-I	pT4N1MX primary tumour	M-72	-	suspension	+	n.d.	+	+
7	SINJ	pT2N1M0 lymph node	M-28	-	suspension	+	+	+	+
8	OEE-III	pT2aN1bM1 lymph node	F/ 53	M918T	adherent	+	n.d.	n.d.	+
9	HEVE-II	pTxN1bMX	F/ 44	-	suspension	+	+	+	+
10	MTC-SK	pT4N1MX lymph node	F/ 51	-	suspension	+	+	+	+

*(Ref. Cote et al. 1986)

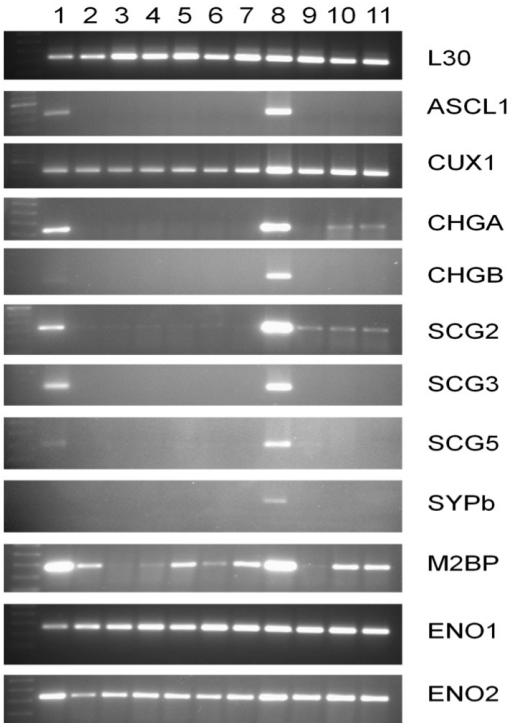

Figure 22. Molecular characterisation of MTC cell lines by RT-PCR. While CUX1, ENO1 and ENO2 are expressed in all cell lines, the expression of other marker genes is heterogeneous among different cell lines. L30, a ribosomal protein served as a positive control.

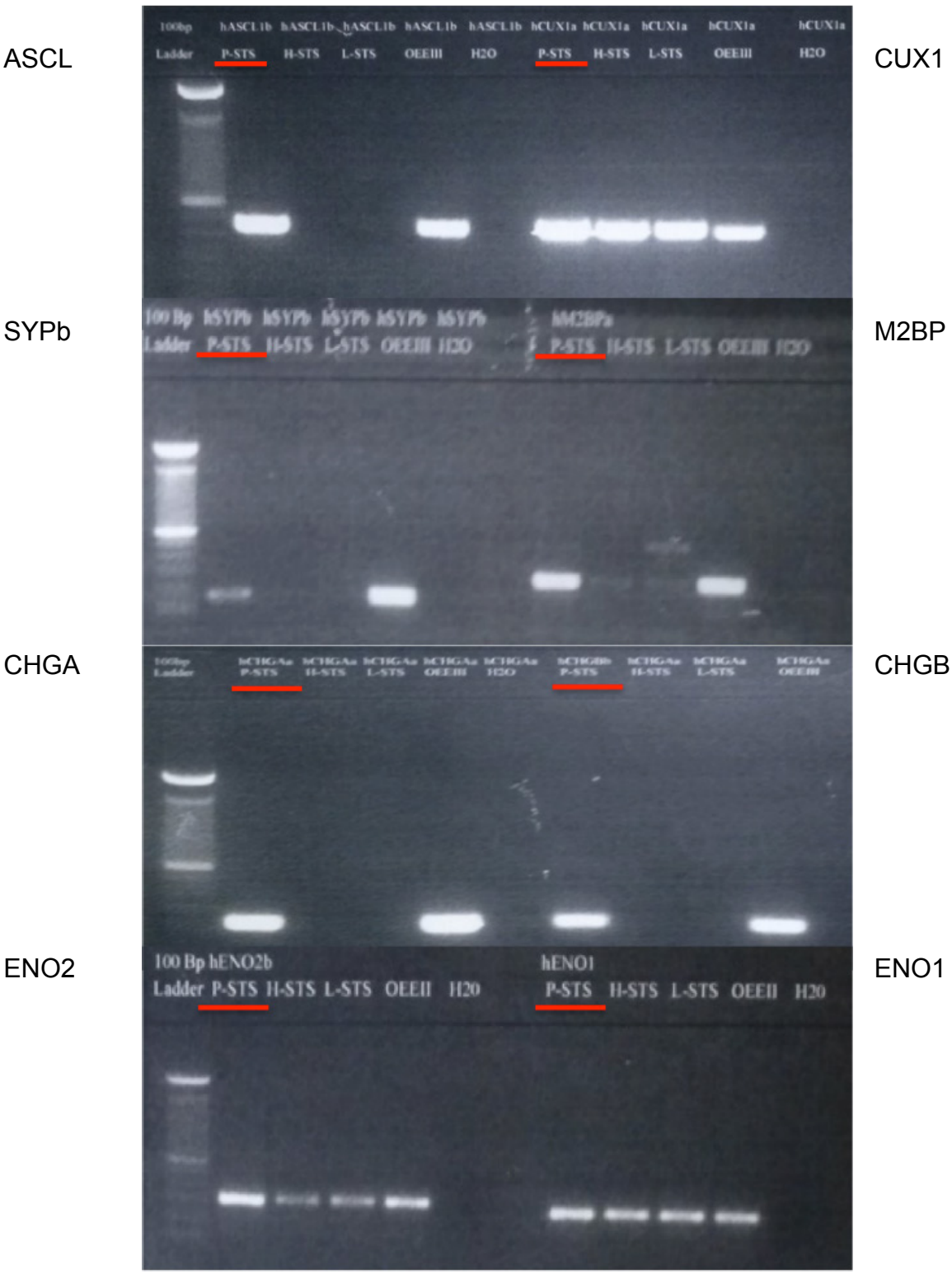

Figure 23. Molecular characterisation of P-STS by RT-PCR. Electrophoresis of marker gene products. P-STS cells (underlined red) express all markers used in this study. (H-STS and L-STS shown on this Figures were not used in this work).

17. Expression Profile of Medullary Thyroid Carcinoma Cell Lines

RT-PCR results from the MTC cell lines are shown in RT-PCR Gel Electrophoresis images and have been additionally analysed by Alpha Digi Doc 100 densitometry for a tabular overview. To edit the results, EXCEL for Mac 2011 was used. The goal was to treat the data, in a qualitative and quantitative manner.

17.1 Expression Analysis of ADAMs in MTC Cell Lines

We examined 14 ADAMs in 10 different human medullary thyroid carcinoma cell lines, plus a later passage of MTC-SK (Figure 24). The intensity of the bands, representing the height of protease expression, was analysed with Alpha Digi Doc 100 software and adapted by the Decision Making Tree (see Materials and Methods – Densitometry). Our results suggest that ADAM 10 was strongly and ubiquitously expressed in all cell lines, whereas ADAM 15, 17, 21, 22 were heterogeneously expressed in all MTC lines. Expression of ADAM 8, 19, 20, 23 could be found in most Medullary Thyroid Carcinoma lines, whether ADAM 9, 28, 32 and 33 showed very little occurrence. Counting bands throughout the cell lines gave us a first overview of how much a protease was expressed in the 10 Medullary Thyroid Carcinoma cell lines. Each band got the numeric value of 1 and was analysed *via* a qualitative approach, summarized by the following box plot (Figure 25).

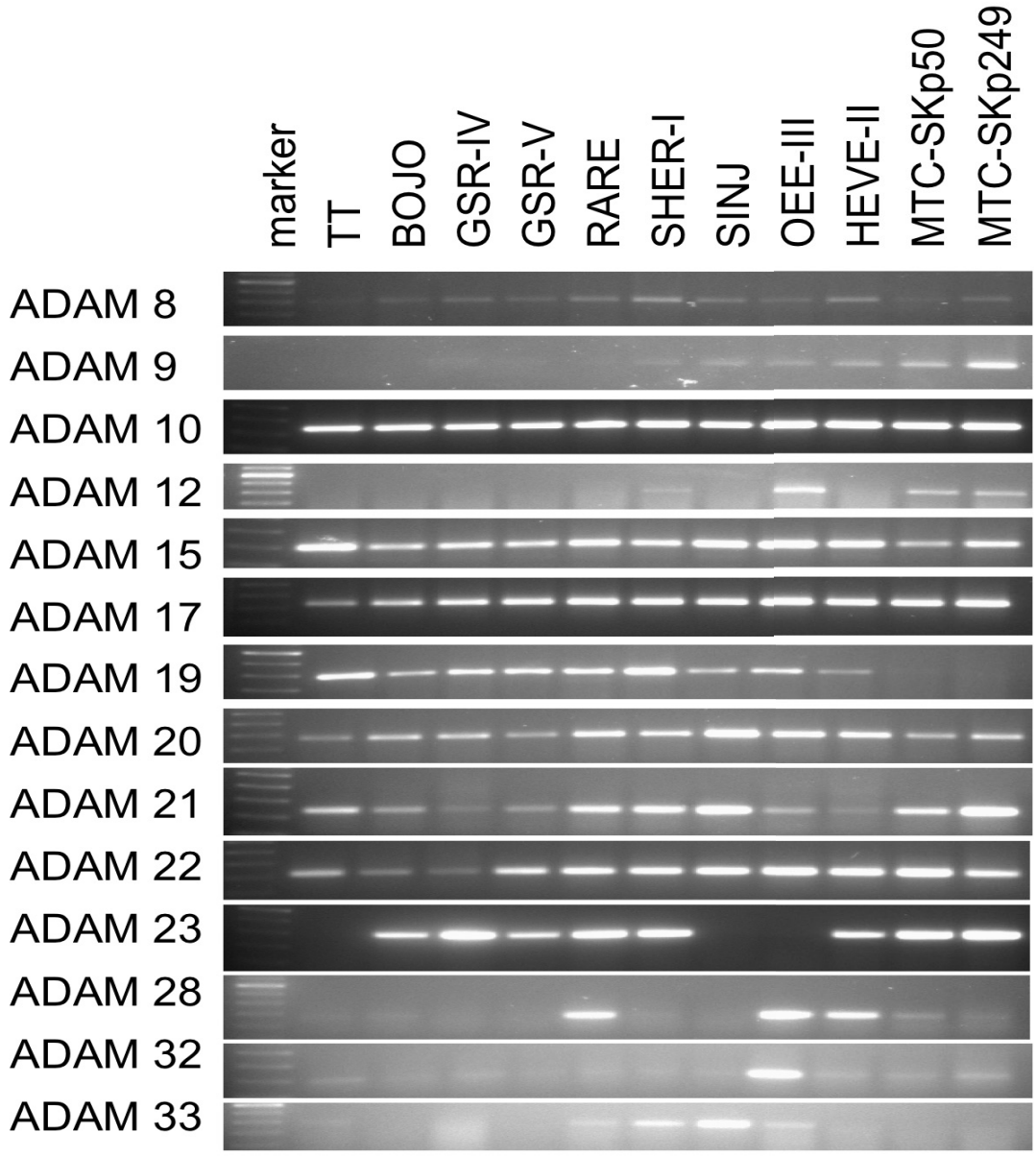

Figure 24. ADAM expression in 10 MTC cell lines. To facilitate the interpretation of the RT-PCR results, only the area where protease expression could be found was cut out of the gel electrophoresis images and stacked upon each other.

Table 16. Quantitative ADAM expression in 10 MTC cell lines

Expression levels of ADAMs in Medullary Thyroid Carcinoma, densitometry results.

	TT	BOJO	GSR-IV	GSR-V	RARE	SHER-I	SINJ	OEE-III	HEVE-II	MTC-SKp50	MTC-SKp249
ADAM 8	0	(+)	(+)	(+)	+	+	(+)	(+)	+	(+)	(+)
ADAM 9	0	0	0	0	0	(+)	(+)	(+)	(+)	+	++
ADAM 10	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
ADAM 12	0	0	0	0	0	(+)	0	++	0	+	+
ADAM 15	+++	+	++	++	+++	++	+++	+++	+++	+	++
ADAM 17	(+)	+	++	+++	+++	+++	+++	+++	+++	+++	+++
ADAM 19	+++	+	++	++	++	+++	+	+	(+)	0	0
ADAM 20	(+)	+	+	(+)	++	+	+++	++	+	(+)	+
ADAM 21	+	(+)	(+)	(+)	++	++	+++	(+)	(+)	++	+++
ADAM 22	+	(+)	(+)	+	++	++	+++	+++	+++	+++	++
ADAM 23	0	++	+++	++	+++	+++	0	0	++	+++	+++
ADAM 28	0	0	0	0	++	0	0	+++	++	(+)	(+)
ADAM 32	(+)	0	0	0	0	0	0	+++	(+)	(+)	(+)
ADAM 33	(+)	0	0	0	(+)	+	+++	(+)	0	0	0

0 no expression (+) very weak expression + weak expression
++ moderate expression +++ high expression

Figure 25. explains how often ADAM Metalloproteases are expressed in our 10 MTC cell lines. The x scale represents all the tested Medullary Thyroid Carcinoma lines (BOJO, GRS-IV, GRS-V, HEVE-II, MTC-SK, OEE-III, RARE, SHER-1, SINJ, TT), the y scale shows ADAM expression (ADAM x/10). The box represents 50% of the total values, which range between 5 to 10 times expression of ADAM proteases in all MTC lines. The median is at 9, suggesting that 50% of all proteases are expressed 9 or 10 times in all MTC cell lines, which is considered an overall high protease expression. The values representing the lower 50% (median to bottom whisker) vary strongly between 3,5 to 9 times expression in all cell lines. Summarized ADAM expression in MTC cell lines is rather high with the exception of ADAM 12, 28, 32 and 33 (shown in Table 17).

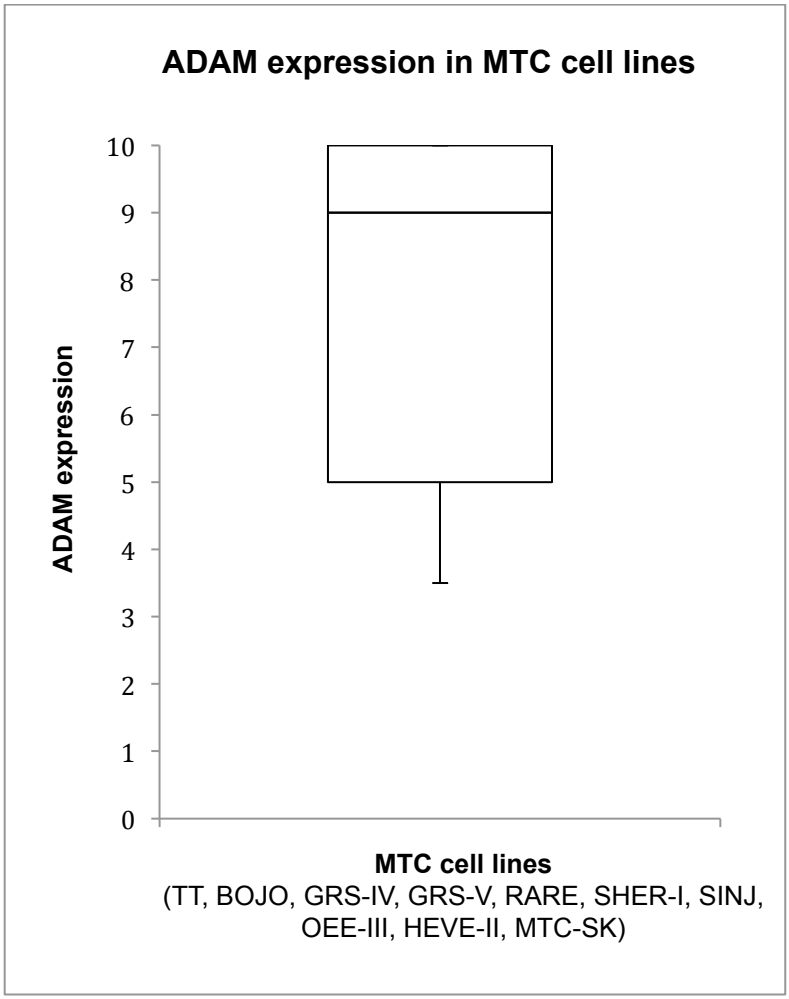

Figure 25. Box blot results of ADAM expression in 10 MTC cell lines

Table 17. Protease expression in all MTC cell lines

Protease	x times expressed in all MTC cell lines (x/10)
ADAM 12	3
ADAM 28	4
ADAM 32	4
ADAM 33	5
ADAM 9	7
ADAM 23	7
ADAM 19	9
ADAM 8	9
ADAM 10	10
ADAM 15	10
ADAM 17	10
ADAM 20	10
ADAM 21	10
ADAM 22	10

For a closer determination of expression intensity we tried to transform the gathered from the densitometry results data by modifying the values of Table 16. We addressed each symbol a numeric value. (+) was awarded 0,5 points, +, 1 point, ++, 2 points and +++, 3 points. In total 30 points could be reached for each protease (3 points per cell line). The enumerated overview is shown in Table 18.

Table 18. Quantitative ADAM expression by points

Protease expression in all MTC lines	(x/30)
ADAM 8	6
ADAM 9	3
ADAM 10	30
ADAM 12	3,5
ADAM 15	23
ADAM 17	24,5
ADAM 19	15,5
ADAM 20	13,5
ADAM 21	12,5
ADAM 22	19
ADAM 23	18
ADAM 28	7,5
ADAM 32	4,5
ADAM 33	5,5

As shown in Table 19., ADAM 8 is expressed 9 times within the 10 MTC cell lines, but gets only 6/30 points, which stands for an overall low expression intensity. A more consistent ratio between qualitative and quantitative results is seen for ADAM 10, 15 and 17. Comparing the results of Table 19., allows a good differentiation between qualitative and quantitative results. Secondary we transformed the punctual score into a percentage scale, to lighten the interpretation.

Table 19. Qualitative and quantitative comparison of ADAM expression in MTCs

Protease expression in 10 MTC lines	qualitative (x /10)	quantitative (x/30)
ADAM 8	9	6
ADAM 9	7	3
ADAM 10	10	30
ADAM 12	3	3,5
ADAM 15	10	23
ADAM 17	10	24,5
ADAM 19	9	15,5
ADAM 20	10	13,5
ADAM 21	10	12,5
ADAM 22	10	19
ADAM 23	7	18
ADAM 28	4	7,5
ADAM 32	4	4,5
ADAM 33	5	5,5

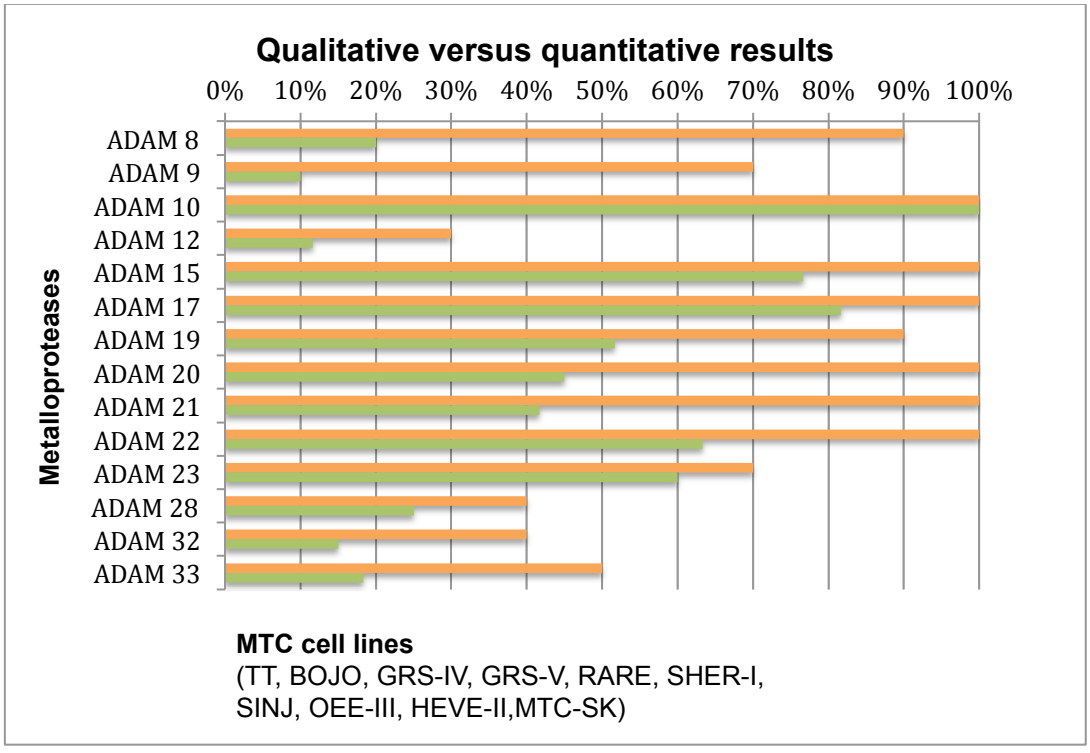

Figure 26. Qualitative (orange) versus quantitative (green) ADAM expression in MTC cell lines

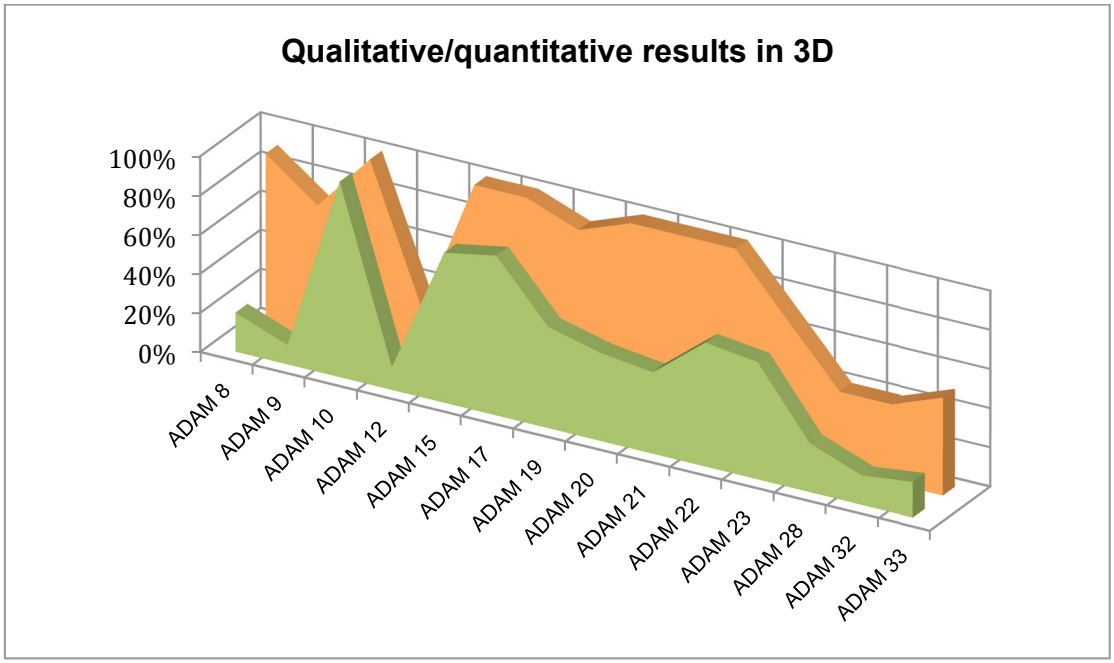

Figure 27. Qualitative (orange = x/10) to quantitative (green = x/30) ADAM expression in MTC (3D)

Not only did we examine the proteases. We also took a close look at ADAM expression for each cell line and examined their expression by a qualitative and quantitative approach. No cell line expressed all 14 ADAMs although enzyme expression was very high (>90%) in OEE-III (13/14), and high (>=80%) in SHER-I (12/14), HEVE II (12/14), RARE (11/14) and MTC-SK (12/14). The lowest expression of a protease per cell line was found in TT, BOJO, GRS-IV and GRS V with 9/14 times expressed.

Table 20. Comparison between qualitative and quantitative results of all ADAMs *per* cell line (numeric)

Cell line	qualitative (x/14)	quantitative (x/42)
TT	9	13
BOJO	9	10,5
GRS-IV	9	14,5
GRS-V	9	14,5
RARE	11	23,5
SHER-I	12	22
SINJ	10	23
OEE-III	13	25
HEVE-II	12	20
MTC-SK	12	19

For a better arrangement of the data we transformed the numeric results into percentage (Table 21.) and displayed qualitative and quantitative values next to each other (seen in Figure 28.).

Table 21. Comparison between qualitative and quantitative results of all ADAMs *per* cell line (%)

Cell line	qualitative (x/14)	quantitative (x/42)
TT	64,3%	31,0%
BOJO	64,3%	25,0%
GRS-IV	64,3%	34,5%
GRS-V	64,3%	34,5%
RARE	78,6%	56,0%
SHER-I	85,7%	52,4%
SINJ	71,4%	54,8%
OEE-III	92,9%	59,5%
HEVE-II	85,7%	47,6%
MTC-SK	85,7%	45,2%

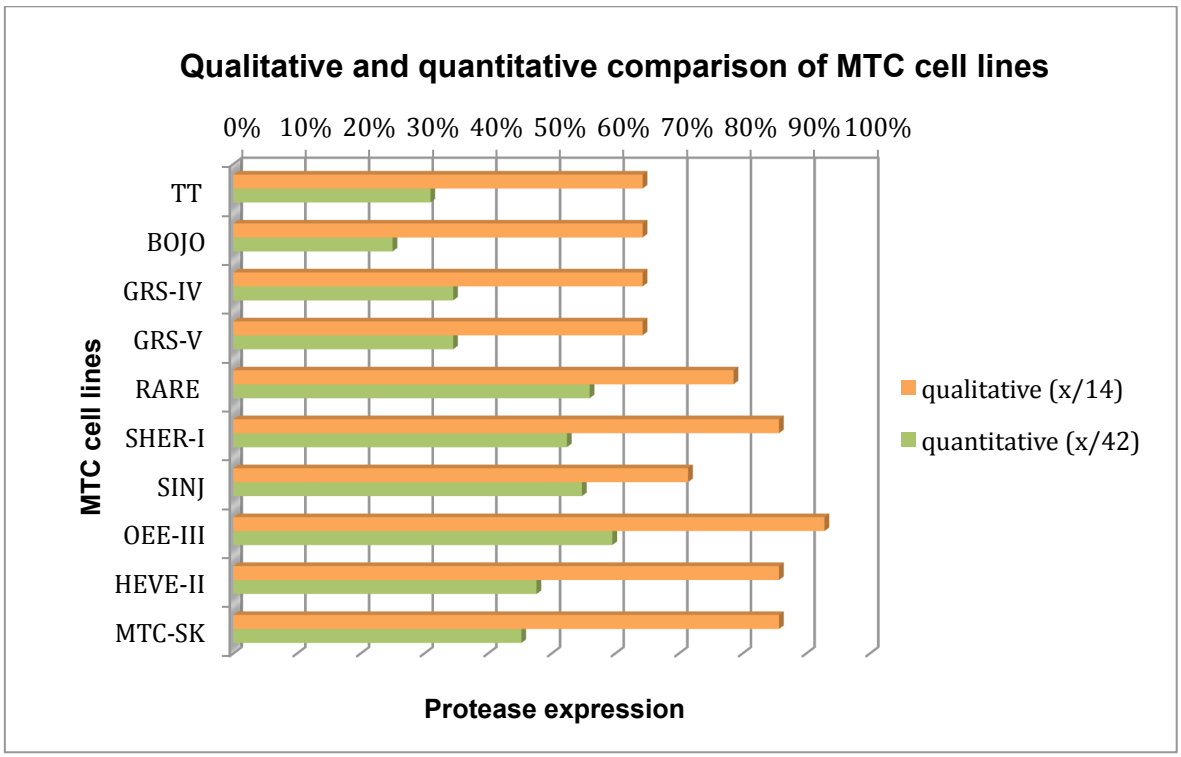

Figure 28. Bar chart analysis of MTC cell lines (qualitative/quantitative results)

17.2 MMP Expression in MTC Cell Lines

Non of Matrix Metalloproteases was expressed in every cell line. The expression of MMP 7 could be found in most of the Medullary Thyroid Carcinoma lines (8/10), while the rest of MMPs showed little occurrence. No cell line expressed all Matrix Metalloproteases and expression was generally found under 30% with the exception of SHER-I (36%).

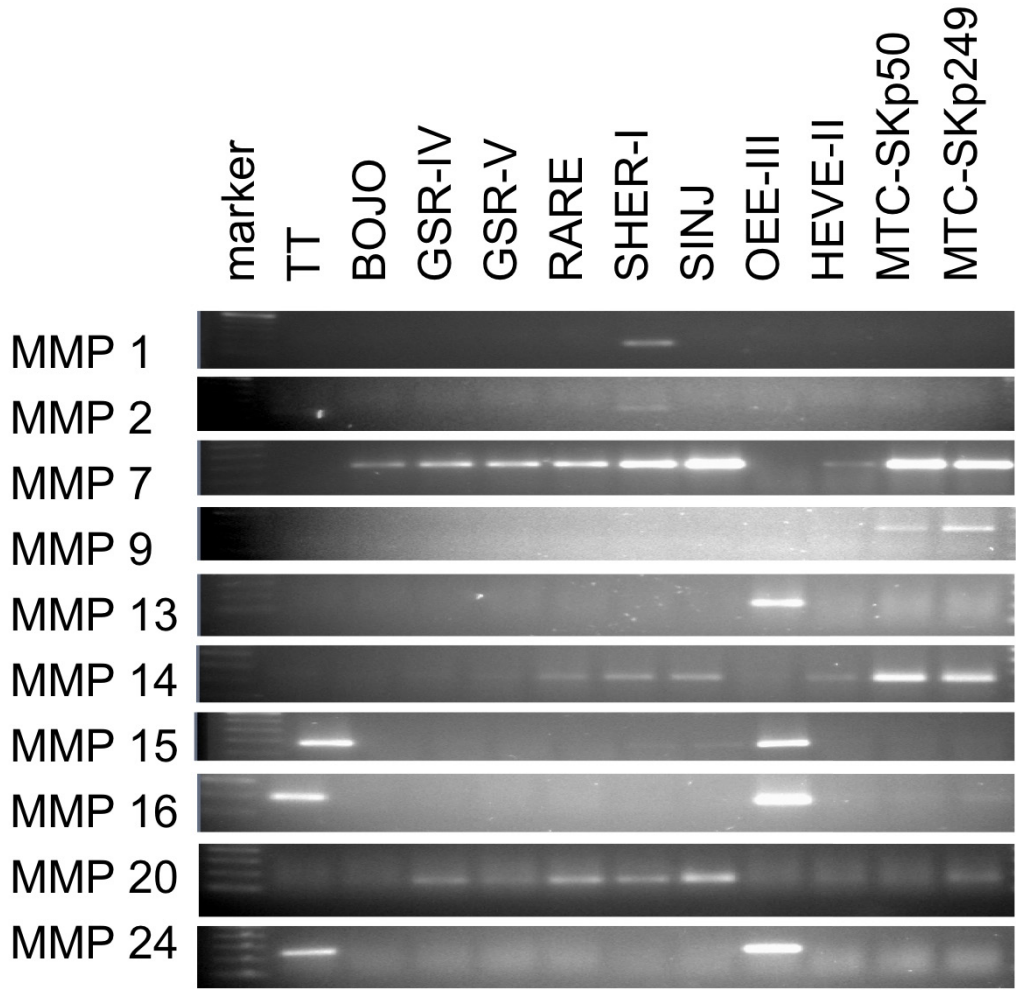

Figure 29. MMP expression in 10 MTC cell lines

Table 22. Quantitative expression of Matrix Metalloproteases in 10 MTC cell lines

Expression levels of MMPs in MTCs

	TT	BOJO	GSR-IV	GSR-V	RARE	SHER-I	SINJ	OEE-III	HEVE-II	MTC-SKp50	MTC-SKp249
MMP 1	0	0	0	0	0	+	0	0	0	0	0
MMP 2	0	0	0	0	0	(+)	0	0	0	0	0
MMP 7	0	(+)	+	+	+	++	+++	0	(+)	+++	++
MMP 9	0	0	0	0	0	0	0	0	0	(+)	(+)
MMP 13	0	0	0	0	0	0	0	++	0	0	0
MMP 14	0	0	0	0	(+)	+	+	0	(+)	++	++
MMP 15	++	0	0	0	0	(+)	(+)	++	0	0	0
MMP 16	+	0	0	0	0	0	0	++	0	0	0
MMP 20	0	0	(+)	(+)	(+)	(+)	+	0	0	0	0
MMP 24	+	0	0	0	0	0	0	++	0	0	0

0 no expression to (+) very weak expression + weak expression ++ moderate expression
+++ high expression

Figure 30. explains how often Matrix Metalloproteases are expressed within the 10 MTC cell lines (MMP x/10). The x scale represents all tested Medullary Thyroid Carcinoma cell lines (BOJO, GRS-IV, GRS-V, HEVE-II, MTC-SK, OEE-III, RARE, SHER-1, SINJ, TT) and the y scale enumerates Metalloprotease expression. The lower 50% of the values range between 1 and 2 times MMP expression in all cell lines (x/10). The median is at 2, describing a low expression for half of the tested Matrix Metalloproteases. The upper 50% of the values vary in a broader range, showing MMP expression between 2 and 8 times (MMP 7) within the tested lines. Summarized, MMP expression in MTC cell lines is rather low with the exception of MMP 7 and MMP 14 and varies quite largely in the upper 50%, where the lower 50% of the values show a more consistent enzyme expression.

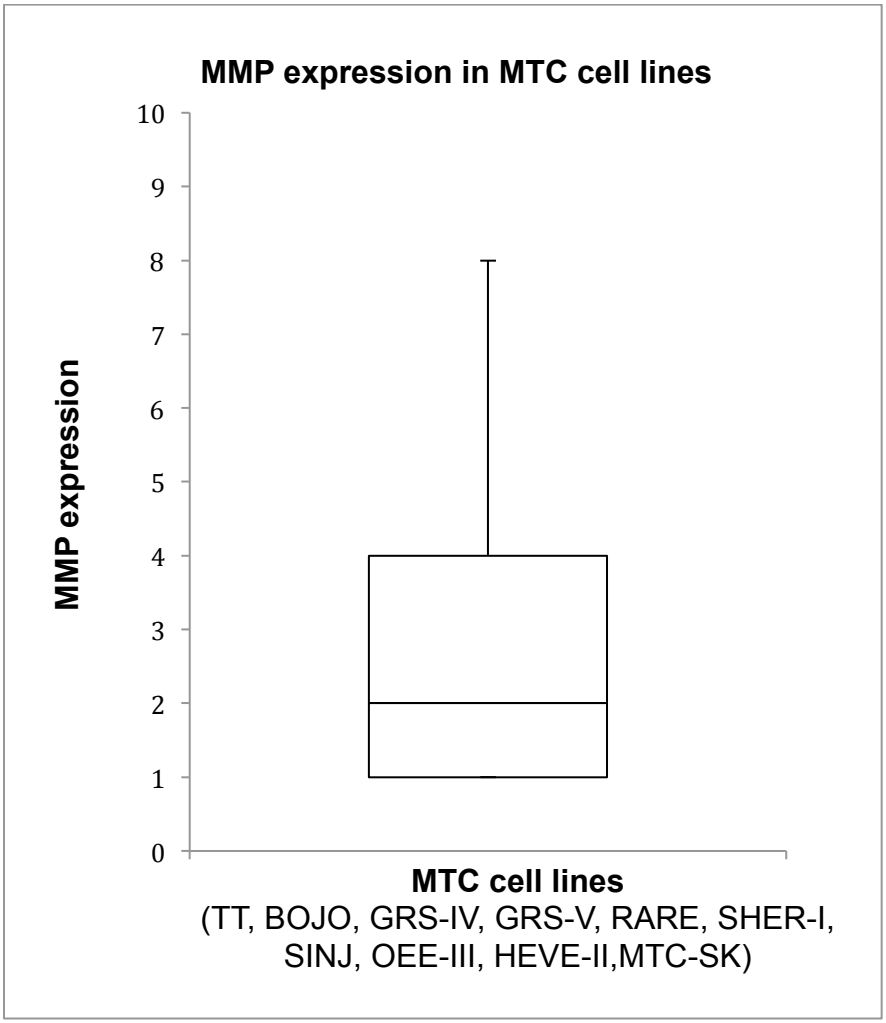

Figure 30. Box blot of MMP expression in 10 MTC cell lines

Table 23. Qualitative and quantitative comparison of MMPs in 10 MTC cell lines

Protease expression in 10 MTC lines	qualitative (x/10)	quantitative (x/30)
MMP 1	1	1
MMP 2	1	0,5
MMP 7	8	12
MMP 9	1	0,5
MMP 13	1	2
MMP 14	5	5
MMP 15	4	5
MMP 16	2	3
MMP 20	4	3
MMP 24	2	3

Again we transformed the numeric score into a % scale to ease interpretation.
As shown in Table 24., MMP expression in the tested MTC cell lines is rather low, with the exception of MMP 7, that is expressed in 8 of 10 cell lines. But the expression intensity of MMP 7 with 12/30 points (40%) is considerably low. The rest of the tested Matrix Metalloproteases shows expression activity below 17%. 1,7% expression is the lowest value represented by both MMP 9 and 2. We could find a strong negative correlation between the number of expressed proteases and the intensity of these enzymes. A graphical account, shown in Figure 31. and 32., offers an improved summary of Matrix Metalloprotease expression in the 10 MTC cell lines.

Table 24. Qualitative and quantitative comparison of MMPs in 10 MTC cell lines in %

Protease expression in 10 MTC lines	qualitative (x/10)	quantitative (x/30)
MMP 1	10,0%	3,3%
MMP 2	10,0%	1,7%
MMP 7	80,0%	40,0%
MMP 9	10,0%	1,7%
MMP 13	10,0%	6,7%
MMP 14	50,0%	16,7%
MMP 15	40,0%	16,7%
MMP 16	20,0%	10,0%
MMP 20	40,0%	10,0%
MMP 24	20,0%	10,0%

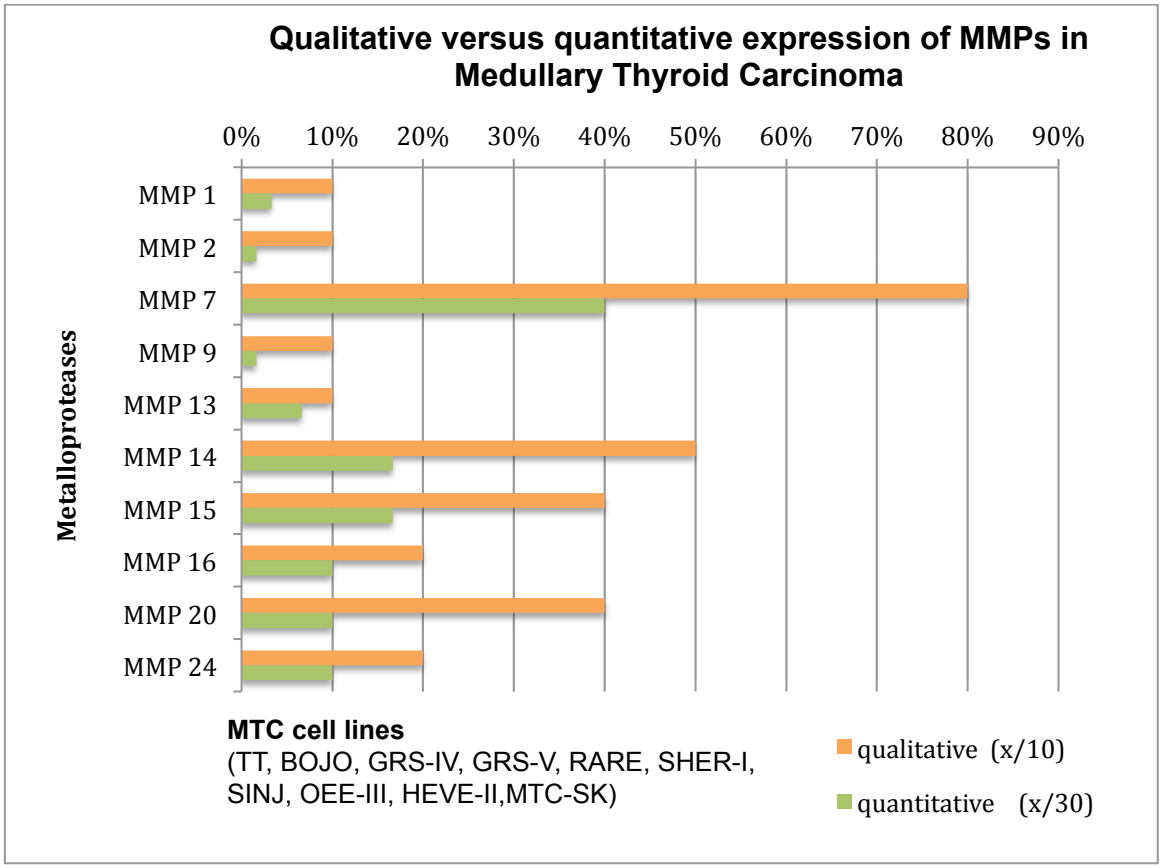

Figure 31. Qualitative versus quantitative expression of MMPs in Medullary Thyroid Carcinoma

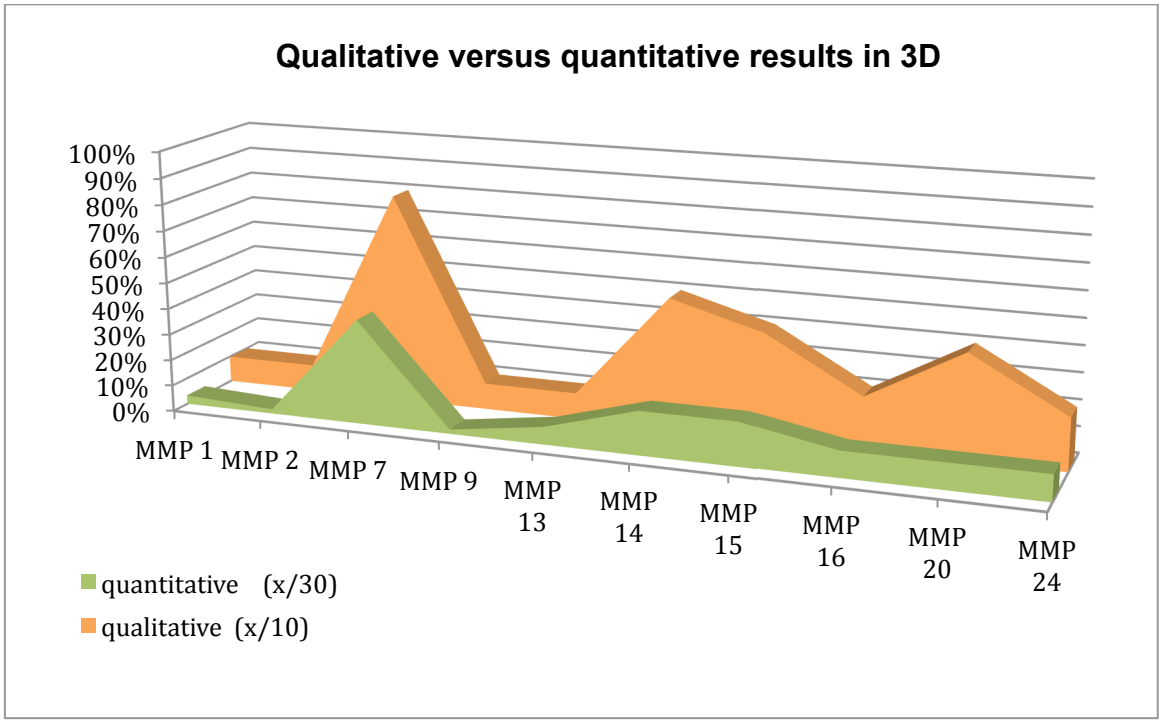

Figure 32. Qualitative versus quantitative expression of MMPs in Medullary Thyroid Carcinoma (3D)

Not only did we examine the proteases. We also took a close look at MMP expression in a certain cell line and examined the expression by a qualitative and quantitative approach. No cell line expressed all MMPs and expression was considerably low, with most of the proteases not reaching more than 30%. SHER-I had the highest enzyme expression with 60% and in SINJ and OEE-II 40% of all MMPs were expressed. The lowest expression of all MMPs per cell line was 10%, in BOJO.

Table 25. Comparison between qualitative and quantitative results of all MMPs *per* cell line

Cell line	qualitative (x/14)	quantitative (x/42)
TT	3	4
BOJO	1	0,5
GRS-IV	2	1,5
GRS-V	2	1,5
RARE	3	2
SHER-I	6	4,5
SINJ	4	5,5
OEE-III	4	8
HEVE-II	2	1
MTC-SK	3	5,5

For a better arrangement of the data, we transformed the numeric results into percentage (Table 26.) and displayed qualitative and quantitative values next to each other (seen in Figure 34.).

Table 26. Comparison between qualitative and quantitative results of all MMPs per cell line (%)

Cell line	qualitative (x/14)	quantitative (x/42)
TT	21 %	9,5%
BOJO	7 %	1,2%
GRS-IV	14 %	3,6%
GRS-V	14 %	3,6%
RARE	21 %	4,8%
SHER-I	43 %	10,7%
SINJ	29 %	13,1%
OEE-III	29%	19,0%
HEVE-II	14%	2,4%
MTC-SK	21%	13,1%

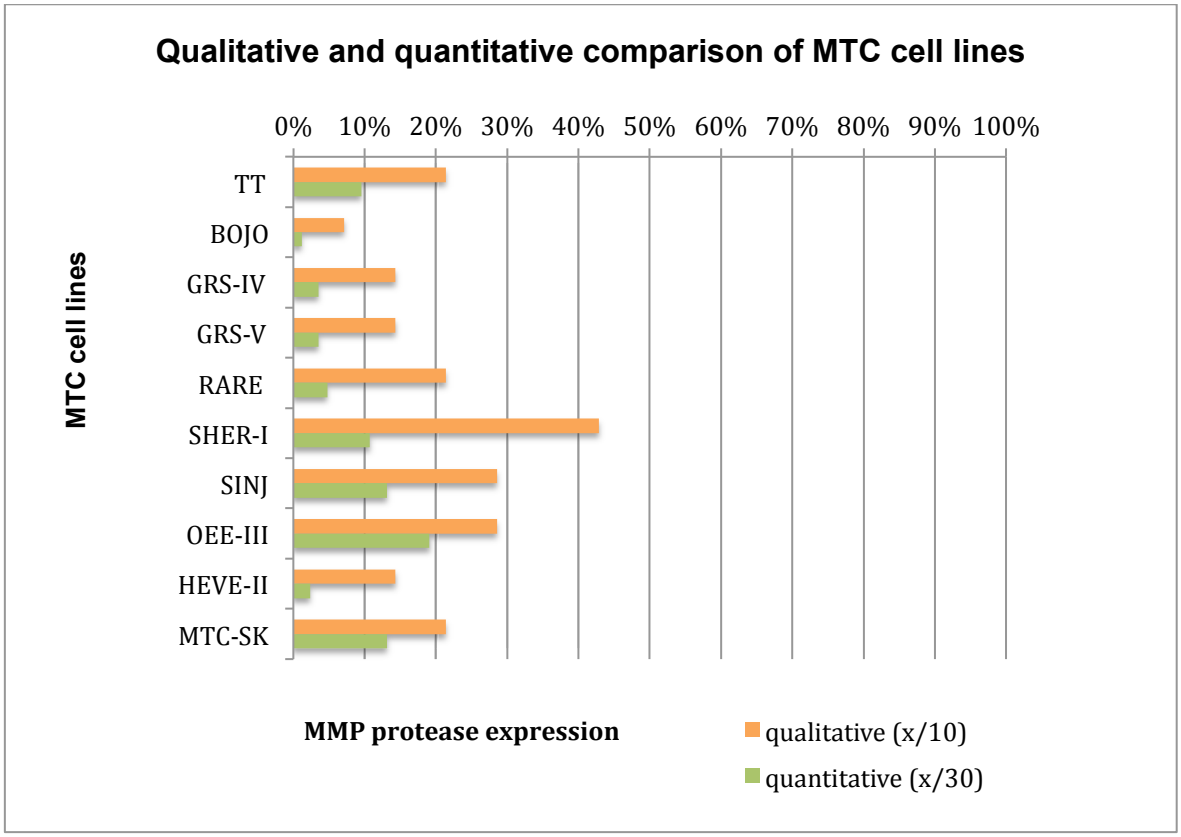

Figure 33. Qualitative and quantitative comparison of MMP expression per MTC cell line

18. Expression Profile of Small Intestine Neuroendocrine Tumour Cell Lines

Additionally to the expression profile for Medullary Thyroid Carcinoma cell lines, we examined 7 chosen ADAMs and 7 MMPs in 2 different human Small Intestine Neuroendocrine Tumour Cell Lines (SI-NETs) P-STS, KRJ-Ip7 and KRJ-Ip95 (additional charge) were used for this approach (Figure 35.), although we based our calculations on P-STS and KRJ-Ip7. Secondary the intensity of the bands, representing protease expression intensity, was remodelled and analysed with Alpha Digi Doc 100 and adapted by the “Decision Making Tree” (see Materials and Methods – Densitometry). Our results claim strong ADAM 9 expression, reaching over 80%. 9 proteases were expressed in both SI-NET lines (ADAM 2, 8, 9, 10, 15, 22 and MMP 2, 7, 17) but showed comparatively low expression intensity

(expect ADAM 9 and MMP 2). No expression was detected for ADAM 32, MMP 15 and 24 in KRJ-Ip7, as well as for MMP 19, 23 in P-STS. Summarized, we found an heterogenous qualitative expression of ADAMs in most of both cell lines, but low intensity, represented by the quantitative values with the exception for ADAM 9. The Matrix Metalloproteases expression intensity in SI-NETs was similar to ADAMs and is considered rather low. A comparison between qualitative and quantitative results is displayed in Figure 36.

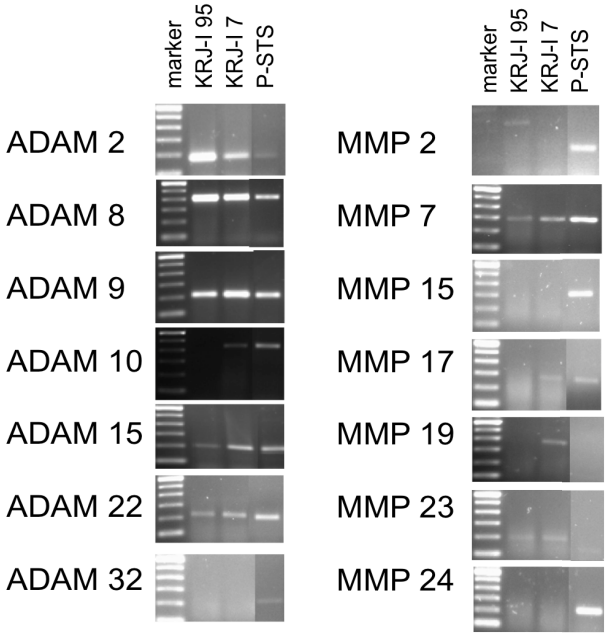

Figure 34. ADAM and MMP expression in SI-NET cell lines. To facilitate the interpretation of the RT-PCR results, only the area where protease expression could be found was cut out of the gel electrophoresis images and stacked together to ease interpretation. KRJ-Ip95 was not taken into account for our analysis.

Table 27. Tabular summary of ADAM and MMP expression in SI-NET cell lines

	KRJ-I p7	P-STS
ADAM 2	+	(+)
ADAM 8	++	+
ADAM 9	+++	++
ADAM 10	(+)	+
ADAM 15	+	+
ADAM 22	+	++
ADAM 32	0	(+)
MMP 2	+	+++
MMP 7	+	++
MMP 15	0	++
MMP 17	(+)	(+)
MMP 19	+	0
MMP 23	(+)	0
MMP 24	0	+++

0 no expression, (+) very weak expression + weak expression

++ moderate expression +++ high expression

Table 28. Qualitative and quantitative comparison of ADAMs and MMPs in SI-NETs cell lines (P-STS and KRJ-Ip7)

Protease expression in SI-NETs	qualitative (x/2)	quantitative (x/6)
ADAM 2	2	1,5
ADAM 8	2	3
ADAM 9	2	5
ADAM 10	2	1,5
ADAM 15	2	2
ADAM 32	1	0,5
MMP 2	2	4
MMP 7	2	3
MMP 15	1	2
MMP 17	2	1
MMP 19	1	1
MMP 23	1	0,5
MMP 24	1	3

Table 29. Qualitative and quantitative comparison of ADAMs and MMPs in SI-NET (P-STS and KRJ-Ip7) cell lines (%)

Protease expression in SI-NETs	qualitative (x/2)	quantitative (x/6)
ADAM 2	100%	25%
ADAM 8	100%	50%
ADAM 9	100%	83%
ADAM 10	100%	25%
ADAM 15	100%	33%
ADAM 32	50%	8%
MMP 2	100%	67%
MMP 7	100%	50%
MMP 15	50%	33%
MMP 17	100%	17%
MMP 19	50%	17%
MMP 23	50%	8%
MMP 24	50%	50%

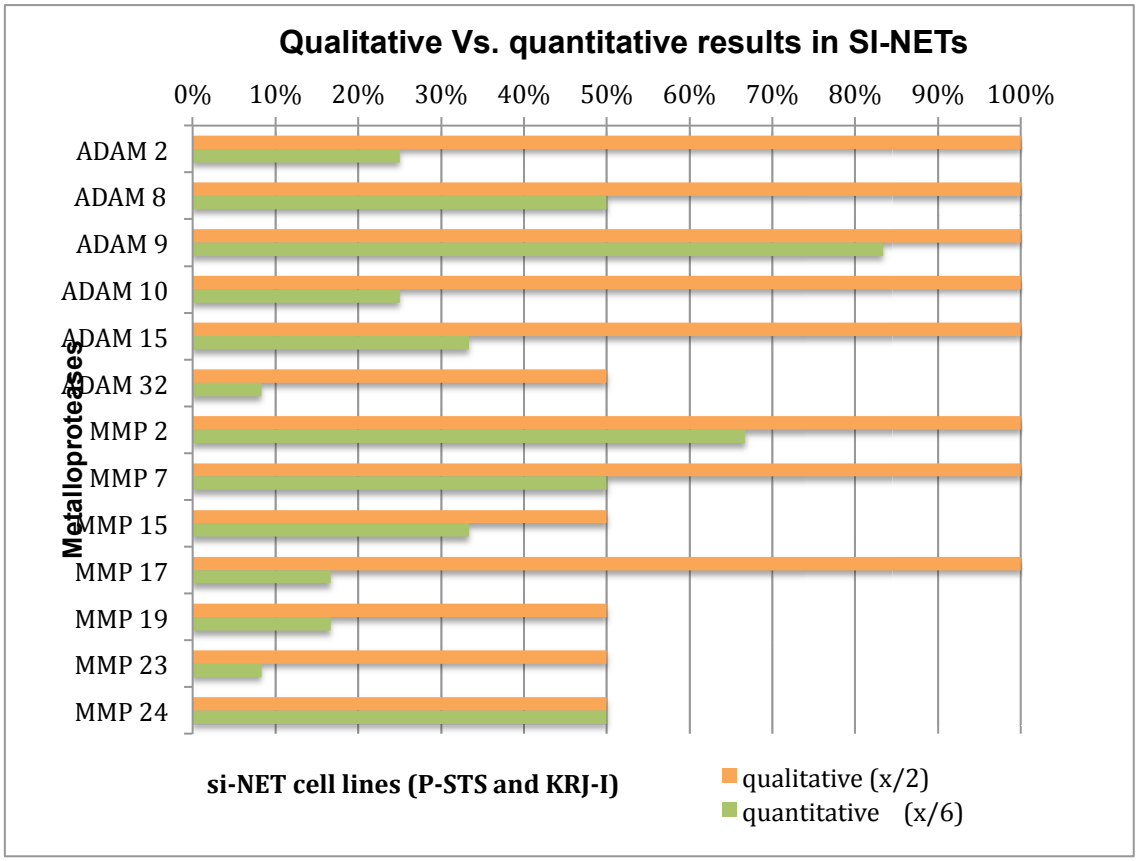

Figure 35. Qualitative and quantitative confrontation of Metalloproteases (ADAMs and MMPs) in SI-NET cell lines P-STS and KRJ-Ip7.

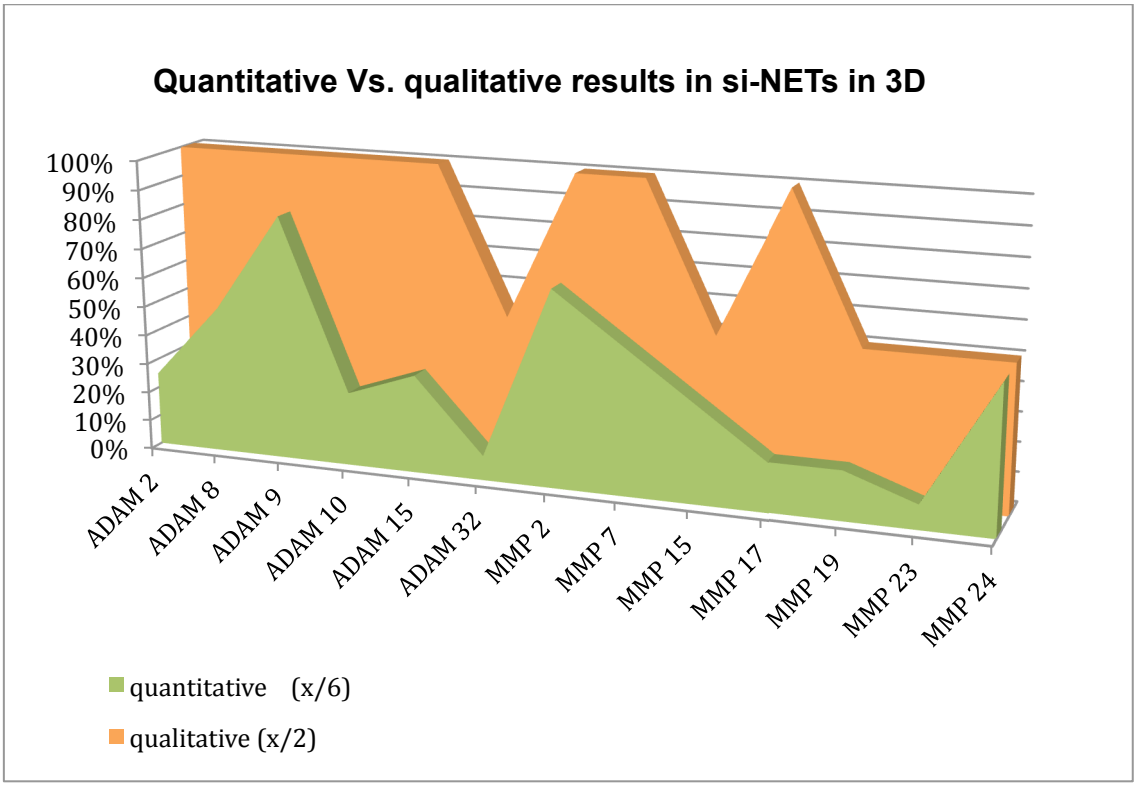

Figure 36. Graphical interpretation of ADAM and MMP expression in SI-NET cell lines (P-STS and KRJ-Ip7).

Next we examined ADAM and MMP expression per cell line, again in a qualitative and quantitative approach. No cell line expressed all tested Metalloproteases (ADAMs and MMPs) although enzyme expression was high in both cell lines. KRJ-Ip7 showed a slightly lower protease expression. The difference between qualitative and quantitative expression varies strongly also in SI-NET cell lines.

Table 30. Comparison between qualitative and quantitative results of MP expression per cell line

Cell line	qualitative (x/14)	quantitative (x/42)
P-STS	12	18,5
KRJ-Ip7	11	12,5

Again we transformed the numeric data into a percentage scale for a better reproducibility (Table 31.).

Table 31. Comparison between qualitative and quantitative results of MP expression per cell line

(%)

Cell line	qualitative (x/14)	quantitative (x/42)
P-STS	85,7%	44,0%
KRJ-Ip7	78,6%	29,8%

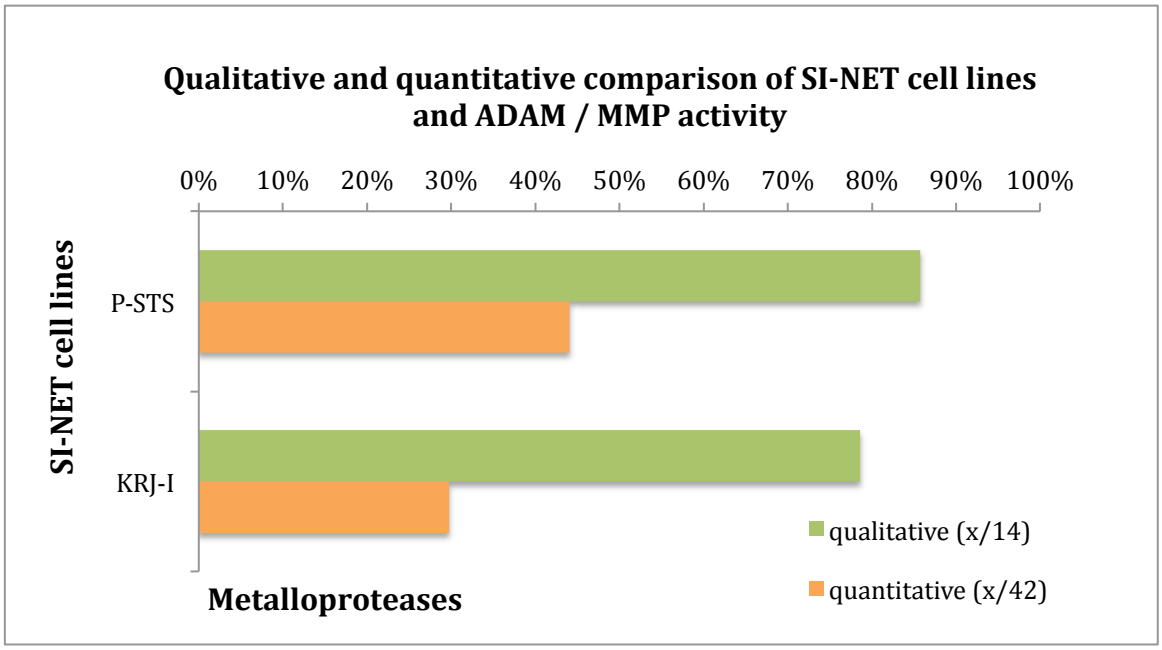

Figure 37. Qualitative and quantitative comparison of ADAM / MMP expression in P-STS and KRJ-Ip7 cell lines

DISCUSSION

Metalloproteases have been identified as key players in the metastatic cascade. Their ability to promote tumour progression, to alter angiogenesis, and to facilitate degradation of the Extracellular Matrix (Noël *et al.* 2012), makes them valuable targets for what is called, a degradomic therapy approach (Gomis-Rüth 2003). MP involvement in malignant processes has already been reported for various human tissues (Shao *et al.* 2014, Zhang *et al.* 2014, Hagemann *et al.* 2012, Schröpfer *et al.* 2010). The influence of ADAM Metalloproteases in the malignant turnover of tumour cells is currently topic to several anti-neoplastic approaches [12]. The establishment of valid expression patterns helps to understand tumour behaviour and defines differences between malignant neoplasms, to optimize treatment and specify detection. Overexpression of ADAM Metalloproteases in different tumours has been already reported for ADAM 8, 9, 10, 12, 15, 17, 19 and 28 (Mochizuki and Okada, 2007). Until now however, little research about the challenging MTC is available, concerning ADAM involvement in its malignant appearance. Anti – ADAMs, such as Anti - ADAM 10 or 17, have been tried in therapeutic approaches and showed positive effects on tumour reduction in mice. Nevertheless clinical trials in humans were stopped, because of the musculoscelettal toxicity of these agents (Saftig and Reiss, 2010). ADAM 17 or TACE (Tumour Necrosis Factor α Converting Enzyme) plays a considerably important role in cleaving the inactive Tumour Necrosis Factor α (TNF- α), a molecule responsible for the activation of other Metalloproteases and the breakdown of the Extracellular Matrix in many tumours (DasGupta *et al.* 2009).

ADAM	Molecular weight (full length, kDa)	Expression in cancers	Functions in cancers	Inhibitors	TIMP inhibition
ADAM8	88.7	Lung,[43] kidney,[68] brain[44]	Promotion of migration	Batimastat (BB94)	None
ADAM9	ADAM9: 90.5 ADAM9s: 72.3	Breast,[45] pancreas[69] Stomach,[50] skin,[70] liver,[71] lung[48]	Promotion of cell adhesion and invasion, binding to integrins (α6β4 and α2β1)	CGS 27023	None
ADAM10	84.1	Oral cavity,[49] stomach,[72] ovary,[51] uterine,[51] colon,[73] leukemia,[74] prostate[75]	L1 shedding, promotion of cell growth and migration	GI254023X, INCB3619	TIMP-1, TIMP-3
ADAM12	ADAM12m: 99.5 ADAM12s: 80.4	Brain,[54] breast,[11] liver,[23] stomach,[50] colon[55]	HB-EGF shedding, promotion of cell growth	KB-R7785	TIMP-3
ADAM15	87.7	Breast,[58] prostate,[76] stomach,[50] lung[57]	Promtion of cell growth	No study	No study
ADAM17	93	Breast,[58] ovary,[77] kidney,[68] colon,[78] prostate[79]	TGF-β shedding, promotion of cell growth	INCB3619	TIMP-2, TIMP-3
ADAM19	105	Brain,[44] kidney[68]	No study	Batimastat (BB94)	None
ADAM28	ADAM28m: 87 ADAM28s: 65	Lung,[62] breast,[63] kidney[68]	IGFBP-3 cleavage, promotion of cell growth	KB-R7785	TIMP-3, TIMP-4

Figure 38. ADAM involvement in cancer (Mochizuki and Okada, 2007)

Matrix Metalloproteases are slightly better investigated than all the other Metalloproteases. MMP 2 is already an established biomarker for metastasis in neuroendocrine tumours (van Veelen *et al.* 2009). However only a couple studies exist, examining the role of MMPs in various human tissues. Some colleagues already provided complete expression patterns for MMPs in specific tumour cell lines but none yet for MTCs (Hageman *et al.* 2012, 2010, Schröpfer *et al.* 2010). Due to a small number of available Medullary Thyroid Carcinoma cell lines, such investigation could not be adequately performed until now. At the Institute of Pathophysiology and Immunology of Graz, known for its pioneering work in the field of MTC cell culture, several Medullary Thyroid Carcinoma cell lines, thanks to Prof. Pfragner and colleagues, could be developed and were made available for this study.

In this work we created expression profiles of A Disintegrin and Metalloproteases (ADAMs) and Matrix Metalloproteases (MMPs) for 10 various Medullary Thyroid Carcinoma cell lines and also for 2 Small Intestine - NET cell lines. Our ADAM selection was chosen by the current findings in literature, which promoted ADAM involvement in cancer. We could demonstrate a rather high expression for all tested A disintegrin and Metalloproteases (ADAM 8, 9, 10, 12, 15, 17, 19, 20, 21, 22, 23, 28, 32, 33) in BOJO, GRS-IV, GRS-V, HEVE-II, MTC-SK, OEE-III, RARE, SHER-1, SINJ, TT Medullary Thyroid Carcinoma cell lines. In specific, ADAM 8

and 12 showed low expression (<30%) whereas the rest of the proteases were heterogeneously higher expressed. Very strong expression (>90%) could be detected for ADAM 8, 10, 15, 17, 20, 21 and 22. Secondary to the proclaimed cancerogenous MMPs (MMP 1, 2, 3, 7, 9, 13, 14) (Deryugina and Quigley 2006), we took the expression of MMP 24 (overexpressed in glioma and gynaecologic cancers) MMP 15, 16 and 20 under closer observation (Hageman *et al.* 2012, Schröpfer *et al.* 2010). Summarized MMP expression was rather low with the exception of MMP 7 and MMP 14 and varied quite largely in our tested MTC cell lines. We could only detect very low MMP 2 expression in SHER-I (existing biomarker for Neuroendocrine Tumours). In the other MTC cell lines and also in different passage numbers (MTC-SKp50 and p249), no significant MMP activation could be found. Besides MMP 7, merely none of the tested Matrix Metalloproteases showed a significant overexpression in Medullary Thyroid Carcinoma cell lines.

Recent studies reported the involvement of ADAM 10 in osteosarcoma (Zhao *et al.* 2014) and hepatocellular cancer (Zhang *et al.* 2014). Secondary the appearance of ADAM 12 in small cell lung cancer has been observed (Shao *et al.* 2014). Overexpressed ADAM 17 could be found in head and neck squamous cell carcinoma (Kamarajan *et al.* 2013) and gastric cancer (Carl-McGrath *et al.* 2005). Interestingly ADAM 7 and 29 showed protective aspects against cancer progression (Noël *et al.* 2012). Our findings of overexpressed ADAM 17 support the role of TACE in malignant processes also for Medullary Thyroid Carcinoma (DasGupta *et al.* 2009). Additionally our results concerning overexpressed ADAM 10 are in accordance with the recent literature (Zhang et al. 2014, Zao et al. 2014). ADAM 10, 15 and 17 were expressed the most in all MTC cell lines and had the highest values in our quantitative analysis. Therefore we proclaim them to be further inverstigated, concerning their possible potential as MTC biomarkers.

The expression of Matrix Metalloproteases in our MTC cell lines showed inverse results for MMP 1 and MMP 2 (very low expression in only 1 out of 10 cell lines), contrary to the works of Cavalheiro (Cavalheiro *et al.* 2008, 2010). Difficulties with cell culture, or primer quality might explain these different results. Only revaluation will tell, whether or not MMP 2 is expressed in MTC cell lines. Cavalheiro

conducted two clinical trials, whereas our results are based on regular cell culture. Therefore we questioned, whether or not a more tumour like environment (3D culture) would change protease expression. In a not published analysis we investigated P-STS (SI-NET) charge 122 in regular (2D) cell culture and 3D Spheroids, and could not find diverse protease expression. Still in the trial and error phase of this method, we cannot draw definite conclusions from these findings.

Outlook

Still surgery remains the only curative option for MTC patients for now. But recently Tyrosine Kinase Receptor Inhibitors (TKIs), such as Vandetanib and Cabozantinib, got approved for advanced Medullary Thyroid Carcinoma therapy by the FDA (U.S. Food and Drug Administration) and are changing the course of treatment and prognosis. Also the Notch, MAPK, Glycogen induced Kinase-3 signalling pathways are currently promising research fields for advanced, specific MTC treatment (Roy *et al.* 2013). At the moment there are 8 active clinical trials, 1 in phase IV, 5 in phase II and 2 in phase I running [12]. The high potential of Metalloprotease inhibitors could not yet translate its satisfying results from the mouse model into clinical trials, though to the high toxicity of the agents. Batimastat, Ilomastat and Marimastat were stopped in phase I or II (first generation, peptide-like inhibitors). But also second generation drugs could not yet show beneficial effects in human trials. Therefore the currently in development third generation of non peptide-like inhibitors, will try to avoid interaction with the physiological effects of Metalloproteases (cytokine and growth factor release) and must present higher selectivity to prohibit side effects (Saftig and Reiss, 2010).

In the field of biomarkers an overall satisfying solution has yet to come. Though calcitonin remains the unchallenged diagnostic marker for MTC, rivalry between the currently existing prognostic markers (Carcino-embryonic antigen (CEA), RET mutations, Desmoplasia and MMP2) remains and is still an open field for new agents. Currently double time calcitonin and CEA values are the best prediction for

tumour progression. However both calcitonin and CEA cannot be considered for preoperative risk stratification (van Veelen *et al.* 2009). ADAM involvement in tumour progression and metastasis might finally provide the missing accurate prognostic link for MTCs and help in the development of more suitable therapy strategies. Our findings promote ADAM 10, 15 and 17 as potential prognostic biomarkers in MTC. Their role and efficiency still needs to be further investigated.

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[11] **Denistometry** (http://rsb.info.nih.gov/nih-image/more-docs/Engineering/ImgEngr.html, 27.10.14, 23:23)

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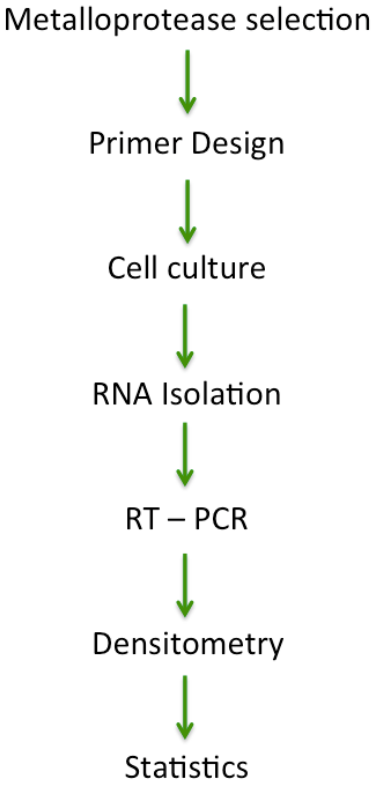
[13] **NET markers** (http://rsb.info.nih.gov/nih-image/more-docs/Engineering/ImgEngr.html, 27.10.14, 23:23)

SUPPLEMENTARY

Project Plan



ADAM 8,9,10,12,15,17,19,28 (Mochizuki and Okada 2007)
MMP 1,2,3,7,9,13,14 (Deryugina and Quigley 2006, Baldini et al. 2004)

ADAMs: 28 (25 genes), MMPs: 26 (23 genes)
(Covering splice variants until August 2013)

10 medullary thyroid carcinoma lines (BOJO, GRS-IV, GRS-V, HEVE-II, MTC-SK, OEE-III, RARE, SHER-1, SINJ, TT)
(Pfragner et al.1990, 2005, Cole et al. 1986)

SOP 20.11.2013

CYCLES						
30	min	50	°C	Reverse transcription		
15	min	95	°C	Hot start activation		
30	sec	94	°C	Denaturation	x	
30	sec	60	°C	Annealing	x	27 times
1	min	72	°C	Elongation	x	
10	min	72	°C	Final elongation		

Alpha Digi Doc 100 + Decision making tree

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