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Trisomies 9 detected by FISH in cutaneous lesions of patients with mastocytosis

Inaugural-Dissertation zur Erlangung der Doktorwürde der Humanmedizin der Medizinischen Fakultät der Universität Bern

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1. Table of Contents

1. Table of Contents 3
2. List of Tables....5
3. List of Figures 6
4. Abbreviations 7
5. Abstract 9
6. Introduction 10
6.1 Mastocytosis....10
6.1.1 Genomic background 10
6.1.1.1 Aberrations of chromosome 8 and 9 in systemic mastocytosis....10
6.1.2 The mast cell 11
6.1.3 Pathophysiology 12
6.1.4 Symptoms....12
6.1.5 Clinical presentation of mastocytosis....13
6.1.5.1 Different forms of cutaneous mastocytosis 14
6.1.5.1.1 Maculopapular cutaneous mastocytosis (Urticaria pigmentosa) 14
6.1.5.1.2 Teleangiectasia macularis eruptiva perstans 19
6.1.5.1.3 Diffuse cutaneous mastocytosis 22
6.1.5.1.4 Solitary and diffuse Mastocytomas of skin 22
6.1.5.2 Different forms of systemic mastocytosis 23
6.1.5.2.1 Different indolent (benign) systemic mastocytosis 23
6.1.5.2.2 Different systemic mastocytosis with associated hematopoietic clonal non-MC lineage disease (SM-AHNMD) 24
6.1.5.2.3 Different aggressive systemic mastocytosis....24
6.1.5.2.4 Mast cell leukaemia....24
6.1.5.2.5 Extracutanenous mast cell neoplasms 24
6.1.6 Diagnosis....25
6.1.7 Therapy 26
6.1.8 Aim of the study....26
7. Laboratory work....27
7.1 Theoretical background....27
7.1.1 Immunofluorescence 27
7.1.2 Fluorescence in Situ Hybridisation (FISH) 29
7.1.2.1 In situ hybridisation....29
7.1.2.2 FISH 29
7.1.2.2.1 Probe 29

7.1.2.2.2 Tissue preparation 29
7.1.2.2.3 Hybridisation 29
7.1.2.2.4 Detection 30
7.2 Materials and methods 32
7.2.1 Clinical characteristics of selected patients 32
7.2.2 Tissues 32
7.2.3 DNA probes for FISH and antibodies for immunofluorescence 32
7.2.4 Double staining: FISH and immunofluorescence 32
7.2.5 Scoring of FISH signals 33
8. Results 34
8.1 Controls 34
8.2 Numerical chromosomal aberrations in skin biopsies of patients with mastocytosis 37
9. Discussion 42
10. Acknowledgments 45
11. References 46

2. List of Tables

Tab. 6-1 Examples of Histamine liberators....12
Tab. 6-2 WHO classification of mastocytosis....13
Tab. 6-3 Diagnostic WHO criteria for systemic mastocytosis....25
Tab. 8-1 Determination of limits for CEP8 by analysing keratinocytes....35
Tab. 8-2 Determination of limits for CEP9 by analysing keratinocytes....36
Tab. 8-3 Determination of trisomy 9 in mast cells....38
Tab. 8-4 Determination of trisomy 8 in mast cells....39

3. List of Figures

Fig. 6-1 Dermal mast cells stained with toluodine blue 11
Fig. 6-2 Positive Darier's sign.....15
Fig. 6-3 Maculopapular cutaneous mastocytosis juvenile form.....16
Fig. 6-4 Mastocytosis bullosa.....17
Fig. 6-5 Maculopapular cutaneous mastocytosis adult form.....18
Fig. 6-6 Extensive maculopapular cutaneous mastocytosis adult form 20
Fig. 6-8 More pronounced progressive variant of TMEP 21
Fig. 6-9 Several mastocytomas in a baby.....22
Fig. 6-10 Single mastocytoma in a baby 23
Fig. 7-1 Schematic presentation of our specific mast cell staining 28
Fig. 7-2 Schematic presentation of FISH for counting chromosome 8 and 9 in our study 31
Fig. 8-1 Mast cells showing trisomies 8 and 9 41

4. Abbreviations

Ab	Antibody
ABL	Abelson murine leukemia viral oncogene homolog
Ab-MuLV	Abelson murine leukemia virus
AML	Acute myeloid leukemia
Asp	Aspartic acid
bcr	Breakpoint cluster region
CD	Cluster of differentiation
CEP	Centromeric enumeration probe
CML	Chronic myeloid leukemia
CT	Computed tomography
DNA	Deoxyribonucleic acid
FGFR	Fibroblast growth factor receptors
FISH	Fluorescence in Situ Hybridisation
FITC	Fluoresceinisothiocyanat
G2-phase	Gap2-phase
IF	Immunfluorescence
Ig	Immunoglobulin
JAK	Janus kinase
LEL	Lymphoepitheliom lymphoma
LOH	Loss of heterozygosity
MC	Mast cell
MDS	Myelodysplastic syndrome
MPS	Myeloproliferative syndrome
MRI	Magnetic resonance imaging
myc	Myelocytomatosis viral oncogene
N/C!	Not countable
PUVA	Psoralen combined with ultraviolet A (UVA)
SCF	Stem cell factor
SDEV	Standard deviation
SM-AHNMD	Systemic mastocytosis with associated clonal hematopoietic non-mast cell lineage disorder
SO	Spectrum orange
S-phase	Synthesis phase
SSC	Saline-sodium citrate
TMEP	Teleangiectasia macularis eruptiva perstans

Val	Valine
WHO	World health organization

5. Abstract

Mastocytosis is a rare disease characterized by proliferation of mast cells in one or several organs. Cytogenetic studies with conventional techniques on bone marrow cells revealed abnormal clones in one third of the patients with systemic mastocytosis. Trisomies of chromosomes 8 and 9 have been reported in myeloproliferative disorders and have been discussed in systemic mastocytosis. The aim of the present study was to examine the presence of numerical aberrations of chromosomes 8 and 9 in skin biopsies of patients with cutaneous mastocytosis. Numerical chromosomal aberrations for chromosomes 8 and 9 were analyzed using a combined FISH- and immunofluorescence staining technique with specific probes for the centromere region of chromosomes 8 or 9 and an anti-human mast cell tryptase antibody in skin biopsies of 16 patients with cutaneous mastocytosis. The most frequent chromosomal aberration was trisomy 9 which was detected in 5 of 7 cases (71 %). Trisomy 8 was only found in 1 of 15 cases (7 %). Our study is the first to demonstrate the feasibility of the double-staining technique with FISH and a cell specific marker for the detection of numerical chromosomal aberrations in a specifc cell population in paraffin-embedded tissue samples in cutaneous mastocytosis. It further shows that numerical chromosomal aberrations of mast cells are a frequent finding (overall 38 %, 6 of 16 cases examined) in skin biopsies of patients with this disease.

6. Introduction

6.1 Mastocytosis

The term mastocytosis denotes a heterogenous group of rare disorders characterised by abnormal growth and accumulation of mast cells in one or several organs [1]. Mastocytosis may occur at any age. Cutaneous mast cell disease is most common in children. It may be present at birth, and 80 % of afflicted children demonstrate lesions by 6 months of age. There is no sex predilection reported for cutaneous mastocytosis. Systemic mastocytosis is generally diagnosed after the third decade of life with a reported male to female ratio of 1:3. The skin is the only involved organ in cutaneous mastocytosis with itching teleangiectases and yellow-brown macules, whereas systemic mastocytosis presents a broad range of clinical signs and symptoms depending on the organs involved. The clinical course ranges from chronic, sometimes spontaneously regressing skin lesions to highly aggressive neoplasms with multisystem involvement and short survival times [2].

6.1.1 Genomic background

Recent data suggest that most variants of mastocytosis (at least those with systemic involvement) are clonal disorders, most of them harbouring somatic point mutations of KIT, a protooncogene that encodes the tyrosinase kinase receptor for stem cell factor (SCF). The most commonly observed mutation substitutes Val for Asp at codon 816, which results in spontaneous activation of the KIT protein (SCF receptor). This mutation is seen in the vast majority of adults with systemic mastocytosis. It may also be found in the mast cells of rare cases of paediatric cutaneous mastocytosis, in which case the presentation and course are atypical, but most cases of typical paediatric cutaneous mastocytosis lack the codon 816 mutation. This mutation undoubtedly plays a role in the pathogenesis of mastocytosis, and also offers a target for the development of more specific therapy. In addition, variant mutations have also been reported.
All mast cells contain mast cell tryptase, whereas chymase is demonstrated in a subpopulation. Neoplastic mast cells show a similar antigenic profile, but in contrast to normal mast cells, have been reported to express CD2 and CD25 [3].

6.1.1.1 Aberrations of chromosome 8 and 9 in systemic mastocytosis

Mast cell diseases have recently been classified among hematological malignancies and are proposed to be included in the myeloproliferative disorders [4]. Myeloproliferative disorders showed trisomy 8 and 9 detected by FISH [5, 6]. Cytogenetic studies with conventional techniques on bone marrow cells revealed abnormal cell clones in one third of patients with systemic

mastocytosis [4, 7]. Two studies examined systemic mastocytosis by FISH. One showed trisomy 8 and 9 in peripheral mononuclear blood cells [8], whereas the other reported no numerical aberrations of chromosomes 8 and 9 in bone marrow mast cells [9].

6.1.2 The mast cell

Mast cells evolve from pluripotent CD34+ precursor cells in the bone marrow. In the peripheral blood they circulate as agranular monocytic appearing cells. After migration into tissues they adopt their typical morphology. Mast cells are spindle-like or cubic shaped cells of 8-20 µm in size which exhibit big intracytoplasmatic granules. The granules are metachromatic, that is, they can be stained with alkaline dyes such as toluidine blue and are thereby easily identified in histological sections. In addition, mast cells can be specifically stained by immunohistochemical methods like using antibodies against chymase and tryptase. Mast cells are found primarily in perivascular areas within almost all tissues, thereof most frequently in skin tissue [2, 3].

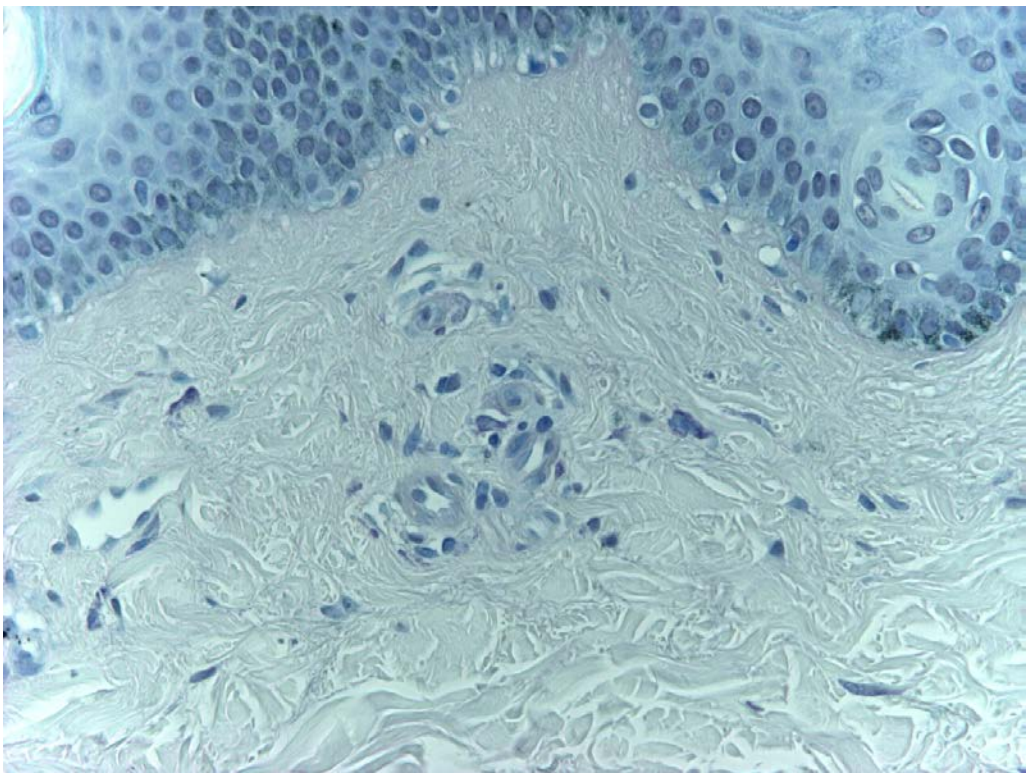

Fig. 6-1 Dermal mast cells stained with toluodine blue. The mast cells are located around the vessels. They contain granules that are stained in dark blue.
(Courtesy of Dr. med. M. Brönnimann)

6.1.3 Pathophysiology

Granules of mast cells contain strong mediators of inflammation such as biogen amines (foremost histamines), proteoglycanes and proteases (especially tryptase and chymase). Typically the granules are emptied if allergenic antibodies interact with IgE-receptors at the surface of the cell. However, many pseudoallergic activators of degranulation, so-called histamine liberators, are known today:

Tab. 6-1 Examples of Histamine liberators [10]

Drugs	Contrast media
	Muscle relaxants (e.g. tubocurarine)
	Analgetics (e.g. morphine, nonsteroidal antiinflammatory drugs, acetylsalicylic acid)
Biological substances and nutrients	Bacterial toxins
	Insect toxins
	Ingredients from seafood
Physical influences	Temperature
	Pressure
	Friction

6.1.4 Symptoms

After stimulation of the H1-receptor histamine secretion of mast cells cause vascular dilatation and permeability, contraction of smooth muscles (bronchia, intestine), neutrophil and eosinophil chemotaxis and urtica formation. Via H2-receptor stimulation additional cardiac symptoms, secretion of gastric acid and stimulation of cytotoxic lymphocytes occur. The systemic activation via the H1-receptor leads to symptoms of an immediate allergy e.g. bronchiospasm, hypotension and oedema.

The mediator dependent symptoms of mastocytosis can be divided into an acute and chronic mediator syndrome. The acute mediator symptom is caused by the release of preformed mediators as histamine and proteases (chymase and tryptase). In the case of the chronical mediator syndrome new mediators are formed as arachidonic acid and its metabolites.

In the case of the systemic mastocytosis additional systemic symptoms occur as fever, adynamia and weight loss and indications of organ infiltration namely lymphadenopathy, anaemia, leucocytopenia, bone marrow fibrosis, osteolysis, fractures [11].

6.1.5 Clinical presentation of mastocytosis

Tab. 6-2 WHO classification of mastocytosis, adapted at [12]

	Variants	Subvariants
Cutaneous mastocytosis		Maculopapular cutaneous mastocytosis (Urticaria pigmentosa)
		Diffuse cutaneous mastocytosis
		Mastocytoma of skin (Mast cell sarcoma of skin)
Systemic mastocytosis	Indolent systemic mastocytosis	Smouldering systemic mastocytosis
		Isolated bone marrow mastocytosis
	Systemic mastocytosis with associated clonal hematologic non–mast cell lineage disease	
	Aggressive systemic mastocytosis	Lymphadenopathic systemic mastocytosis with eosinophilia
	Mast cell leukemia	Typical mast cell leukemia
		Aleukemic mast cell leukemia
	(Extracoutaneous) mast cell sarkoma	
	Extracoutaneous mastocytoma	

6.1.5.1 Different forms of cutaneous mastocytosis

6.1.5.1.1 Maculopapular cutaneous mastocytosis (Urticaria pigmentosa)

Maculopapular cutaneous mastocytosis is the most frequently occuring form of mastosytosis. It is chararctized by red-brownish diffuse confined papulomacullous efflorescences (they measure a centimeter or less in diameter) which cause pruritus. The lesions are most numerous on the trunk and proximal extremities. Irritation of the affected area (e.g. by rubbing the skin) leads to the formation of hives (Darier's sign is positive). The Darier’s sign is an important clinical sign for the diagnosis of cutanous mastocytosis.

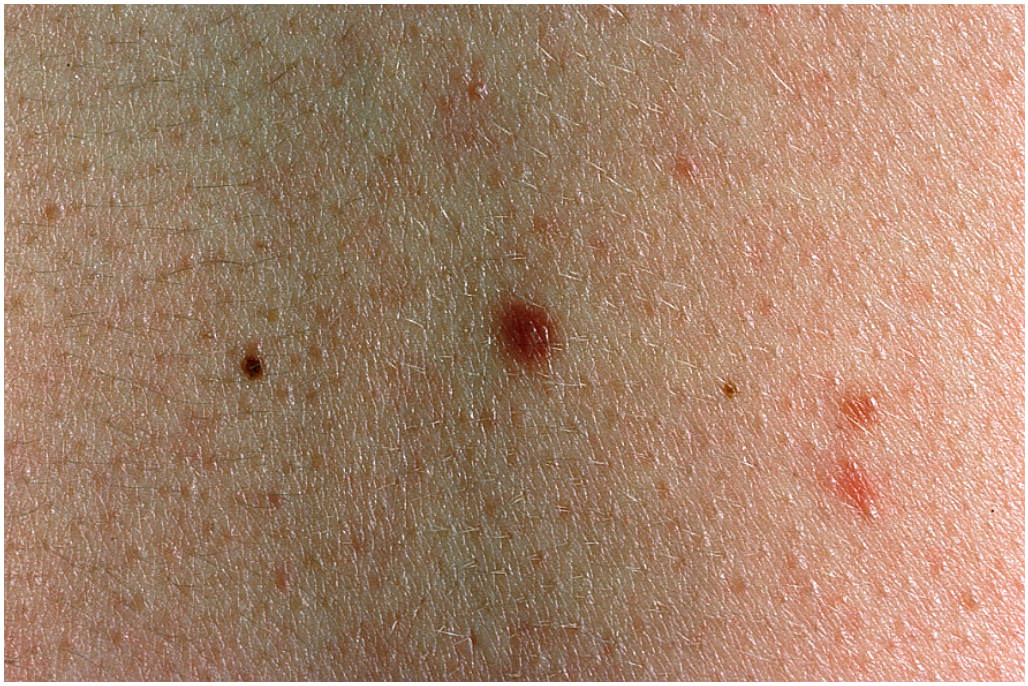

Fig. 6-2a Before rubbing the skin

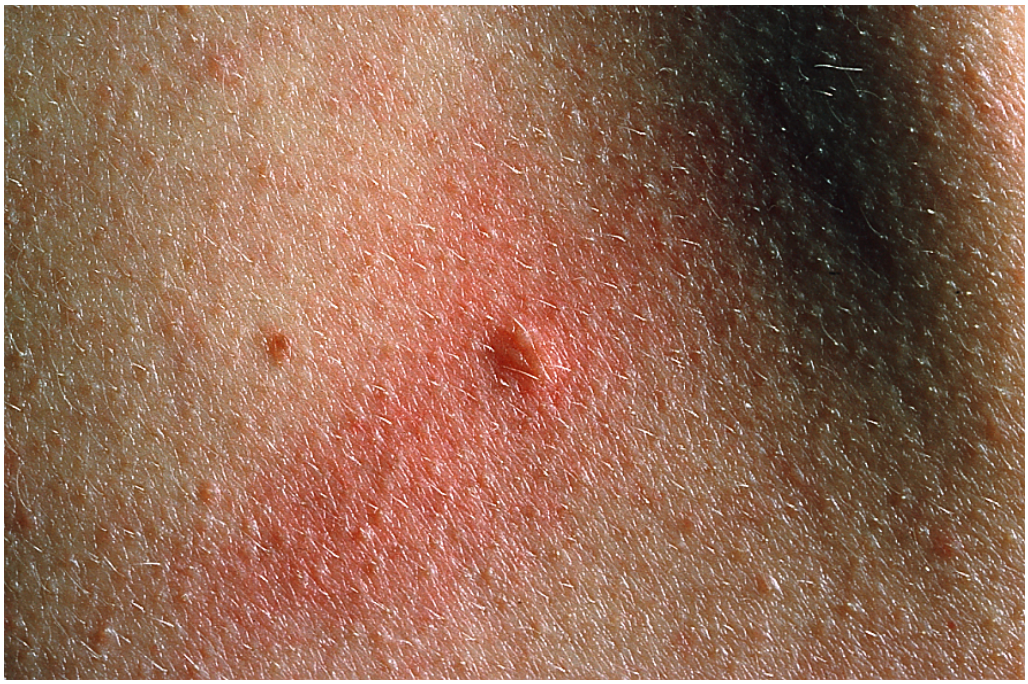

Fig. 6-2b After rubbing the skin

Fig. 6-2a and Fig. 6-2b Positive Darier's sign. The Darier's sign is an important clinical sign for the diagnosis of cutanous mastocytosis, which is typical for cutanous lesions of mastocytosis and a important diagnostic sign: Irritation of the affected area (e.g. by rubbing the skin) leads to the formation of hives (Courtesy of Department of Dermatology, University Hospital Inselspital)

Maculopapular cutaneous mastocytosis is divided into a juvenile and an adult form. The juvenil maculopapular cutaneous mastocytosis rarely affects inner organs and is self-limited. Blisters may develop spontaneously or after scratching, then the maculopapular cutaneous mastocytosis is called mastocytosis bullosa.

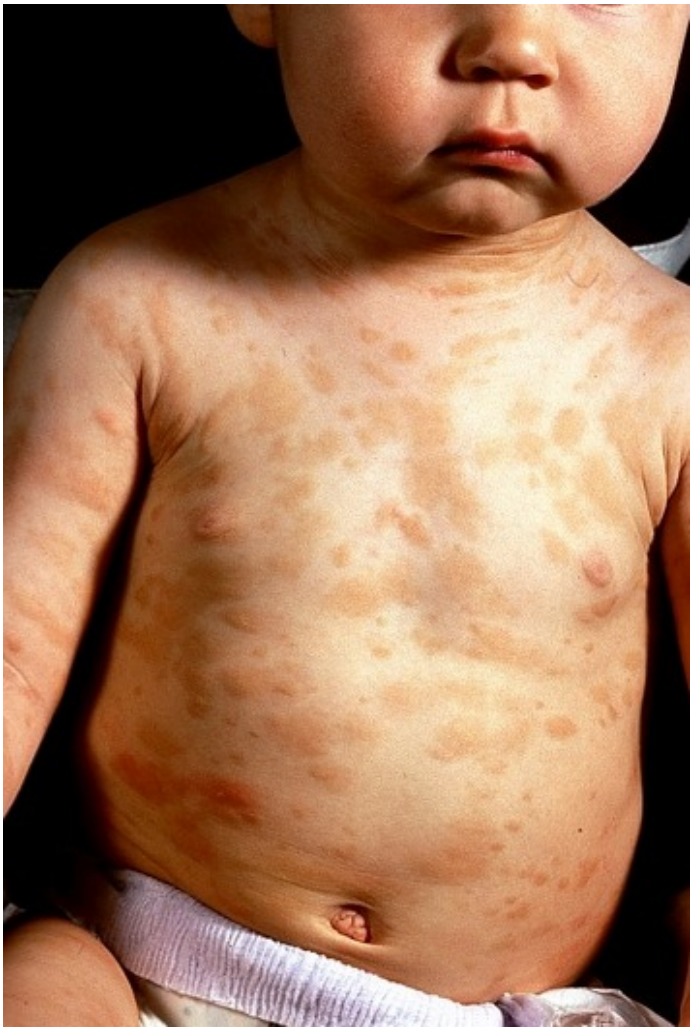

Fig. 6-3 Maculopapular cutaneous mastocytosis juvenile form with brown-red confluent, partially urticarial macules (Courtesy of Department of Dermatology, University Hospital Inselspital)

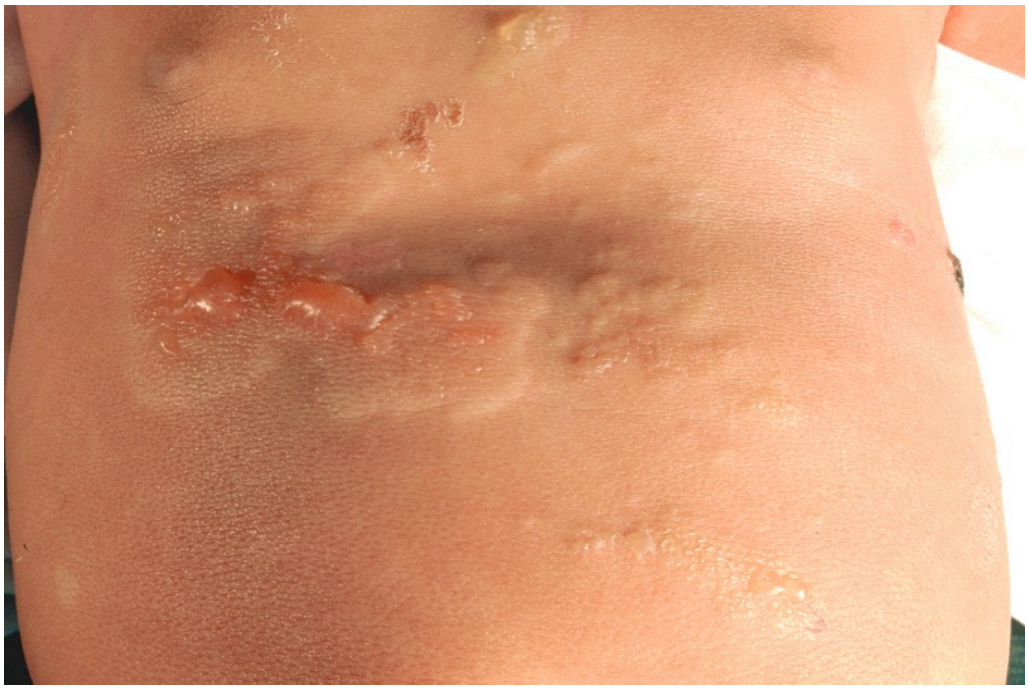

Fig. 6-4 Mastocytosis bullosa. It occurs in babies and shows blisters, often because of scratching but also spontaneously (Courtesy of Department of Dermatology, University Hospital Inselspital)

The adult maculopapular cutaneous mastocytosis can be persistent or chronic progressive. In 30-70 % of the cases the adult mastocytosis is systemic.

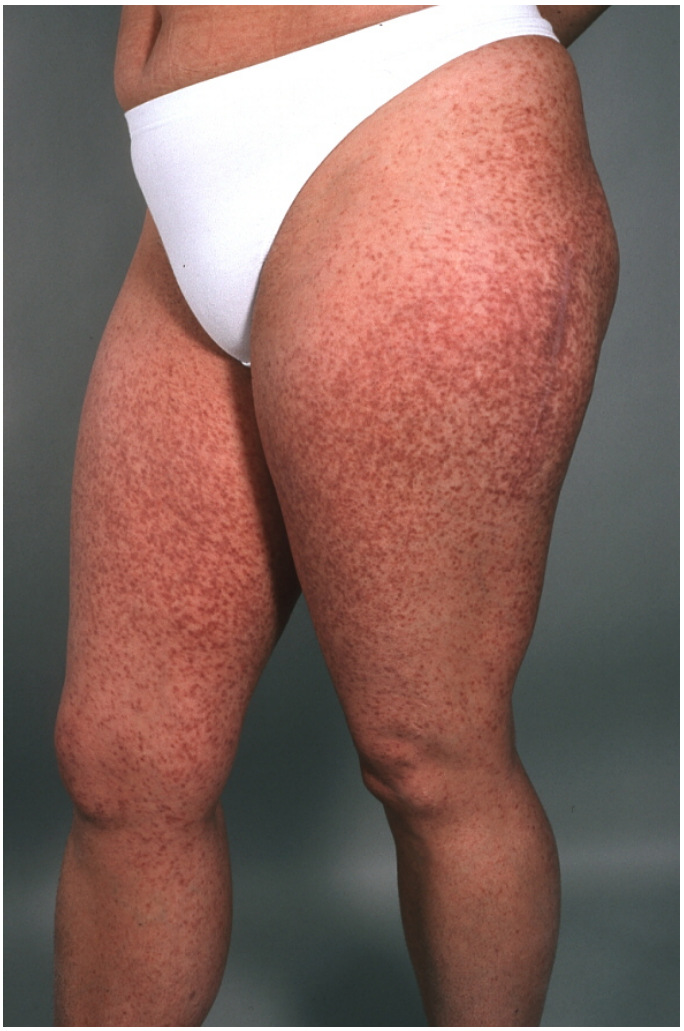

Fig. 6-5 Maculopapular cutaneous mastocytosis adult form with the typical red-brownish, diffuse, sometimes confluent macules in a predilection site on the lower extremities (Courtesy of Department of Dermatology, University Hospital Inselspital)

Fig. 6-6 Extensive maculopapular cutaneous mastocytosis adult form with confluent brown-red macules at typical sites on the trunk and the extremities (Courtesy of Department of Dermatology, University Hospital Inselspital)

6.1.5.1.2 Teleangiectasia macularis eruptiva perstans

A more rare form of adult cutaneous mastocytosis is teleangectasia macularis eruptiva perstans (TMEP) which is characterized by macules and patches composed of telenagiectasias without significant hyperpigmentation. Because of minimal variant pictures it may be difficult to diagnose.

Fig. 6-7 Mild form of TMEP with discrete teleangiectasis and barely visible hyperpigmentation (Courtesy of Department of Dermatology, University Hospital Inselspital)

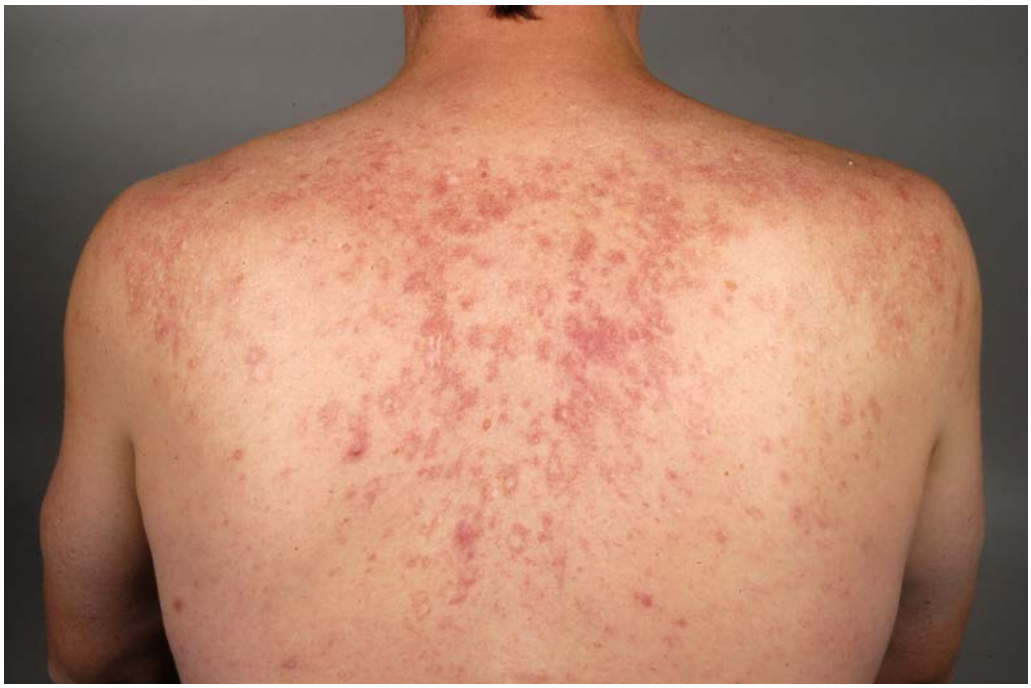

Fig. 6-8a

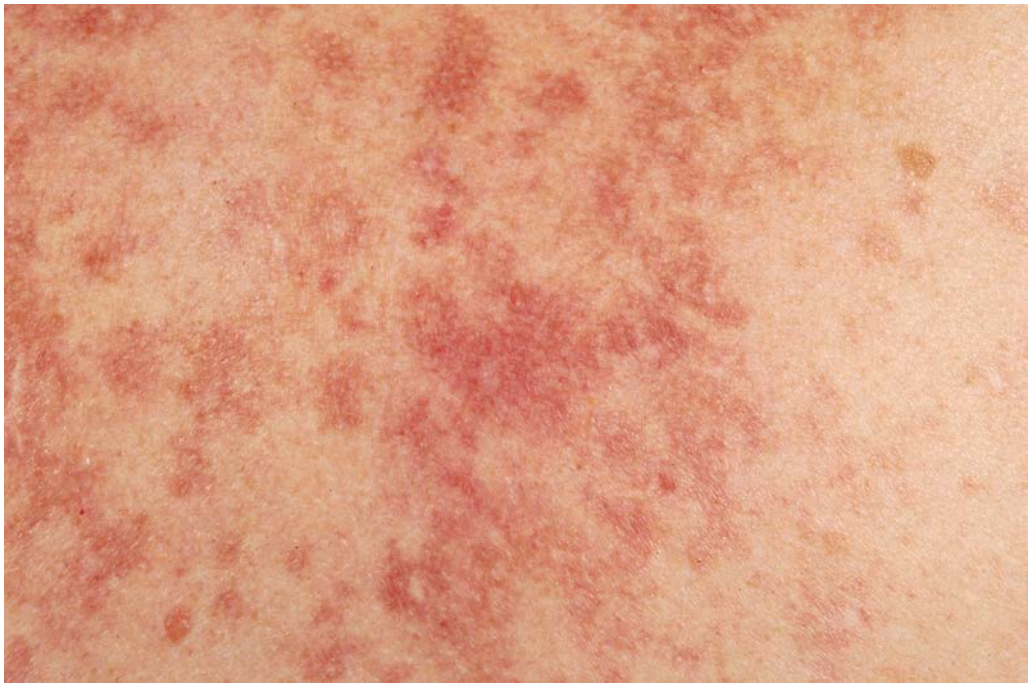

Fig. 6-8b Detail of Fig. 6-8a

Fig. 6-8a and Fig. 6-8b More pronounced progressive variant of TMEP with extensive confluent macular skin efflorescences caused by multiple teleangectasias. No visible pigmentation of the affected skin (Courtesy of Department of Dermatology, University Hospital Inselspital)

6.1.5.1.3 Diffuse cutaneous mastocytosis

Diffuse cutaneous mastocytosis is a very rare form of the disease. It occurs mostly in babies. An erythroderma is formed which is diffusly edematous, thickend and coarsened. Dermographismus is very common and frequently associated with formation of blisters. This form of mastocytosis often disappears before the age of five.

6.1.5.1.4 Solitary and diffuse Mastocytomas of skin

Mastocytomas are mostly observed in babies. The efflorescences are spots, large (about coin-sized), brown-red to dirty-yellow coloured, sharp confined spots or tumurous infiltrates mostly occurring in extremities, but not at palma or planta. They cause severe pruritus. The Darier's sign is positive. Commonly they degenerate within a few months [2, 11].

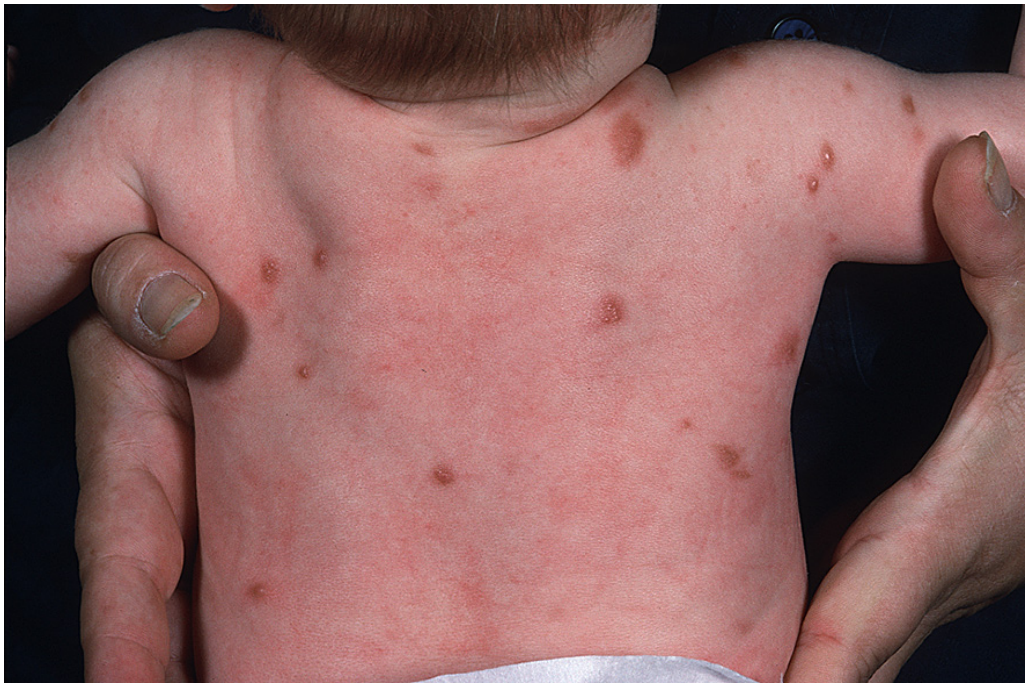

Fig. 6-9 Several mastocytomas in a baby. Multiple brown-red, sharply circumscribed plaques and nodules on the trunk (Courtesy of Department of Dermatology, University Hospital Inselspital)

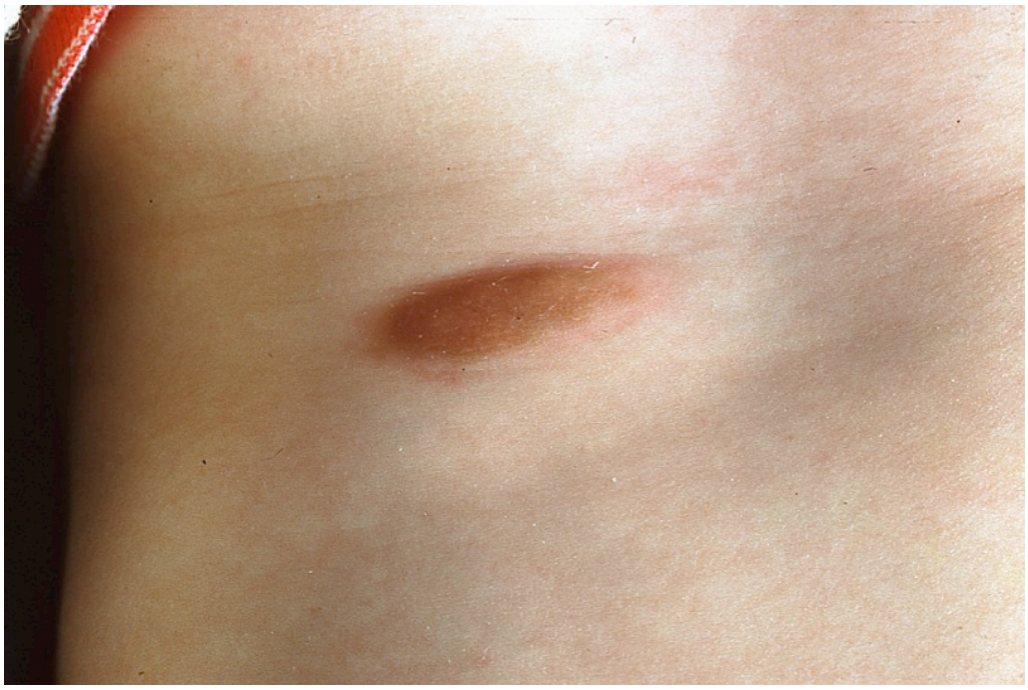

Fig. 6-10 Single mastocytoma in a baby: dirty-yellow plaque, sharp circumcised (Courtesy of Department of Dermatology, University Hospital Inselspital)

6.1.5.2 Different forms of systemic mastocytosis

In systemic mastocytosis mast cell infiltrates are found in the viscera. At least one extracutaneous organ is involved by definition [13, 14]. Systemic mastocytosis is often accompanied by cutaneous skin lesions, which are less abundant in the more aggressive forms.

While most patients with an indolent systemic mastocytosis (and cutaneous mastocytosis as well) can remain in an indolent stage for years to decades, other forms like the agressive systemic mastocytosis SM-AHNMD or mast cell leukaemia can lead to a rapid fatal progression.

6.1.5.2.1 Different indolent (benign) systemic mastocytosis

Indolent (benign) systemic mastocytosis is the most common form of systemic mastocytosis. It accounts for 2/3 of the systemic mastocytosis and is characterised by affection of the skin and bone marrow. For the most parts it shows a prolongated clinical progression with a survival time of two decades and more. Since the diagnosis of the bone marrow infiltration is often difficult to detect immunohistochemical stains (anti-CD25 antibody) and molecular studies (D816V) are required.

Smouldering systemic mastocytosis is a hybrid form of the indolent and the aggressive systemic mastocytosis which often shows tissue infiltrations, serum tryptase level of >200 ng/ml and organomegaly.

The isolated bone marrow mastocytosis is a second subgroup of the indolent systemic mastocytosis and affects the bone marrow exclusively.

6.1.5.2.2 Different systemic mastocytosis with associated hematopoietic clonal non-MC lineage disease (SM-AHNMD)

SM-AHNMD is the second most frequent form of systemic mastocytosis. For the diagnosis the WHO criteria (see below) have to be fulfilled for both systemic mastocytosis and SM-AHNMD. This makes the disease unique among the haematological neoplasms because of the combination of two completely different histologlogies and disease categories. In 80-90 % of the cases it is combined with the following diseases: MDS, MDS/MPS, MPS, AML, LEL and CML [15]. In only 10-20 % it is associated with malignant lymphatic diseases.

6.1.5.2.3 Different aggressive systemic mastocytosis

Aggressive systemic mastocytosis only affects about 5 % of the patients with systemic mastocytosis. Clinically it manifests with hepatosplenomegaly and/or generalised lymphadenopathy. Skin lesions however are very rare. Characteristic for the disease pattern is a progressive infiltration of various organs with consecutive and clinically significant impairment of organ function like cynopenias, malabsorbtion, bone fractures, sings of hepatopathy with loss of liver function. A rare variant of aggressive systemic mastocytosis shows prominent eosinophilia of blood and tissues and generalised lymphadenopaty.

6.1.5.2.4 Mast cell leukaemia

Mast cell leukaemia is the presumably most rare form of human leukaemia. It is characterised by leukaemic infiltration of various organs by immature neoplastic mast cells. In most cases >10 % of circulating blood cells are mast cells.

6.1.5.2.5 Extracutanenous mast cell neoplasms

Extracutaneus mast cell neoplasms are very rare: The mast cell sarcoma as malignant and the extracutaneous mastocytoma as benign extracutaneus mast cell neoplasm [12].

6.1.6 Diagnosis

Along with the typical efflorescences in the cutaneous forms the Darier's sign is nearly pathognomonic: If the efflorescences are irritated an excess of histamine is released causing a localized urticarial lesion. Urticarial dermographism on the affected skin is very common. Obviously, to establish a diagnosis a histopathology should be performed.

The WHO criteria for diagnosis of systemic mast cell disease are shown in Tab. 6-3. In systemic mastocytosis the concentration of mast cell mediators (histamine, tryptase) is increased in plasma and urine. Total tryptase values are recommended by the WHO in the diagnostic evaluation of systemic mastocytosis and values greater than 20 ng/ml represent a minor criterion. However, the significance of an increased tryptase concentration can be deceptive, since it is not only a sign for systemic mastocytosis but can also be existent with cutaneous forms.
If a systemic mastocytosis is suspected a bone marrow biopsy is mandatory. Depending on the clinical pathology an abdominal sonography, an endoscopy of the whole gastric and intestinal tract, a biopsy of the intestinal mucosa, a skeletal radiography, a skeletal szintigraphy, a bone densitometry and combined with headache a CT or MRI has to be performed [2].

Tab. 6-3 Diagnostic WHO criteria for systemic mastocytosis

Major	Multifocal compact infitrates of mast cells in bone marrow or other extracutanous organ(s) (>15 mast cells)
Minor	Mast cells in bone marrow or other extrcutanous organ(s) show an abnormal spindle-shaped morphology (>25 %) c-kit mutation D816V in extracutanous organ(s) (other activating mutation at codon 816 also count as a minor criterion) Mast cells in the bone marrow express CD2 or/and CD 25 Serum tryptase >20ng/ml (does not count in patients who have an associated hematopoietic clonal non-mast cell lineage disease)

If at least one major and one minor criterion or three minor criteria are fulfilled, the diagnosis systemic mastocytosis can be established [12].

6.1.7 Therapy

No curative therapy is available for mastocytosis up to the present. It should be treated according to the symptoms, depending if the disease manifests as cutaneous only or systemic as well.
Single mastocytomas can be excised. Topic glucocorticoids reduce the number of cutaneous mast cells and their histamine content respectively. Furthermore, the PUVA therapy (photochemotherapy) frequently proves to be successful in patients with maculopapular cutaneous mastocytosis. The most commonly used therapy involves antagonists of the H1-receptor. They suppress the pruritus and systemic signs of histamine release symptoms (histamine flush, cardial and gastro intestinal symptoms).
Nifedipine suppresses the histamine flush in systemic mastocytoses. Oral glucosteroids are not a first choice therapy. They are only taken into account in severe cases like diffuse mastocytosis of the skin, distinct disposition of blistering or severe malabsorbtion.
In the case of severe internal progressions the use of cytostatic drugs should be taken into account. Good results were achieved with the use of interferon-α. It suppresses the growth of myeloide progenitor cells. It was reported that in about 15 % of all aggressive systemic mastocytosis interferon-α shows to be effective [16-18].
Furthermore it is very important that patients with symptoms elicit by mast cell mediators are protected from histamine liberators and physical stimuli (e.g. hot or cold baths). Patients who ever experienced an anaphylactic shock should always carry an emergency kit containing ephedrine [11, 12].

6.1.8 Aim of the study

Because biopsies of mastocytosis normally present a diffuse proliferation of mast cells in the involved organs we needed to combine the FISH-examination with a specific marker for mast cells. As shown in other studies mast cell tryptase is a specific marker for mast cells and is also produced in the cells of mastocytosis. The aim of the present study was therefore to establish a technique with an immunohistochemical staining of mast cell tryptase combined with FISH for chromosomes 8 and 9 to specifically detect numerical aberrations of these chromosomes in mast cells of skin biopsies from patients with cutaneous mastocytosis.

7. Laboratory work

7.1 Theoretical background

7.1.1 Immunofluorescence

Immunofluorescence (IF) is a technique, which allows the identification and localization of specific proteins and antigens in cells or tissues using specific chromophore-labeled antibodies. Therefore two methods are used, the direct and the indirect immunofluorescence, respectively. For direct immunofluorescence staining the specific primary antibody is directly labeled with a fluorescent dye; indirect Immunofluorescence uses a non-labeled primary antibody, which is targeted with a chromophore-labeled secondary antibody. The advantage using indirect IF, results an amplification of the signal intensity and allows combining the specific primary antibody with a set of secondary antibodies, increasing the experiment diversity.

Examination of immunofluorescent stained probes is performed using a fluorescence microscope, which allows the excitation of the chromophores and their detection [19].

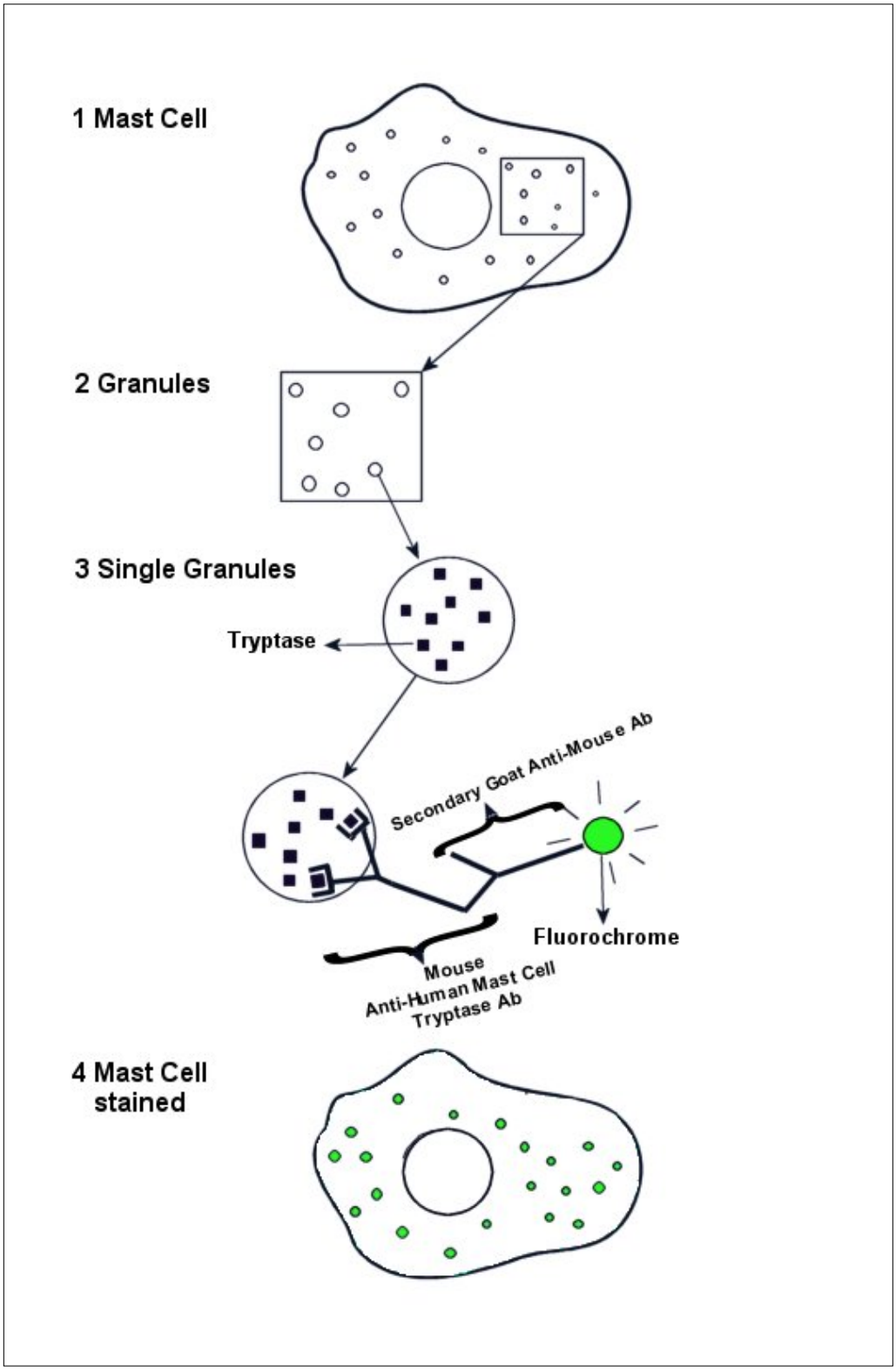

Fig. 7-1 Schematic presentation of our specific mast cell staining: Immunfluorescence with a monoclonal mouse anti-human antibody (ab) FITC (Fluoresceinisothiocyanat)((green) labelled goat anti-mouse Ab

7.1.2 Fluorescence in Situ Hybridisation (FISH)

7.1.2.1 In situ hybridisation

In situ hybridisation is a method, which allows the detection of a nucleic acid sequence at its place in a cell where it is naturally located. The in situ hybridisation relies like other methods (northern- and southern blot) on the Watson/Crick basepairing between a target polynucleodide and a detector polynucleotide with complementary sequence.
First radiolabelled complementary sequences where used for detection [20] followed by elecron microscopy scanning of attached polymer spheres [21]. These days either fluorescent reporter molecules attached indirectly or directly to the sequence specific nucleotides are commonly used [22].

7.1.2.2 FISH

The fluorescence in situ hybridisation comprises of three basic steps: the fixation of the probe, the hybridisation of the homologous DNA fragment and the detection of the tagged target hybrids.

7.1.2.2.1 Probe

The most often used probes are the chromosome specific repetitive DNA sequences. This satellite repeat sequences are located at the centromeres of chromosomes. Via centromer labelling enumerations of chromosomal monosomies or trisomies can be detected very rapidly.

7.1.2.2.2 Tissue preparation

Fluorescence in situ hybridisation can be successfully used on most biological tissues including whole cells, tissue sections, chromosomes and isolated nuclei. The tissues are fixed before hybridisation. In most cases the probe is attached to a glass microscope slide. To increase the efficiency of probe penetration pre-treatments can be applied like proteolytic digestion of the tissue with proteases.

7.1.2.2.3 Hybridisation

The double stranded probe and target DNA has to be denaturated prior to the hybridisation to allow the basepairing of single stranded probe with the target DNA. The DNA double strand can be

dissociated either by heat or alkali denaturation. Temperature and salt concentrations have to be adjusted to get optimum annealing of probe to target.

7.1.2.2.4 Detection

After hybridisation non specifically bound probe has to be removed by washing steps. The hybridized probe can be detected directly if it is coupled to a fluorochrome [20-22].

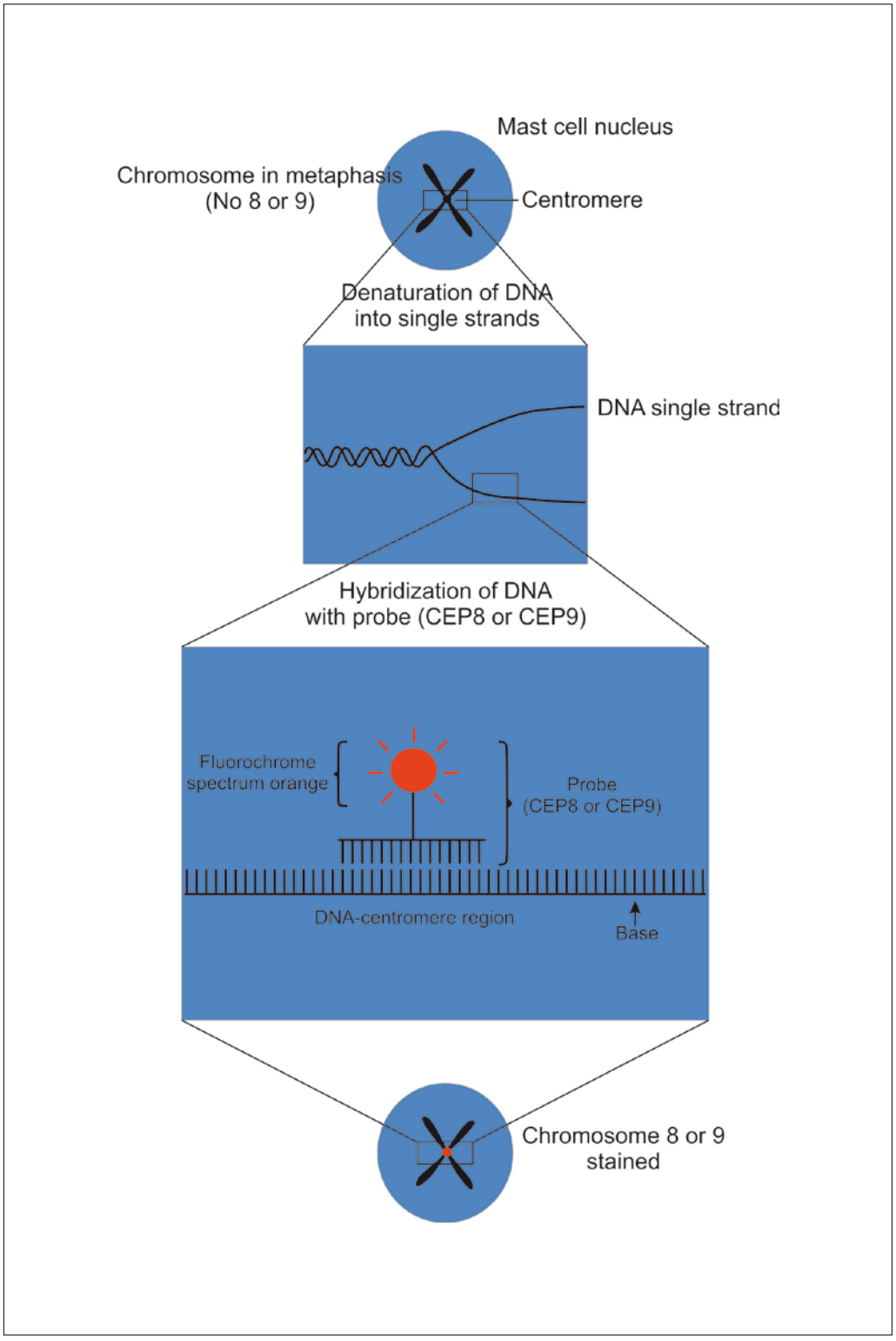

Fig. 7-2 Schematic presentation of FISH for counting chromosome 8 and 9 in our study. The cells were hybridized with a spectrum orange-labelled centromeric enumeration probe (CEP) for chromosome 8 and 9. Each dot in a nucleus corresponds to chromosome 8 or 9

7.2 Materials and methods

7.2.1 Clinical characteristics of selected patients

Before analyzing the paraffin-embedded skin biopsy specimens from 16 patients with cutaneous mastocytosis we thoroughly analyzed their medical history. The average age of the patients was 44 years, ranging from 2 to75 years, with a m:f ratio of 8:8. Diagnosis of cutaneous mastocytosis was based on medical history and clinical findings which did not indicate any signs for systemic involvement. Serum tryptase levels were documented in five patients. In four patients values were below 20 ng/ml. In one patients with a tryptase values of 29,4 ng/ml a bone marrow biopsy was performed which did not indicate any signs of systemic mastocytosis.

7.2.2 Tissues

We analyzed paraffin-embedded skin biopsy specimens from these 16 patients with cutaneous mastocytosis. Histological sections were re-examined and the skin lesions classified according to the WHO system. Tissue arrays with 9 to 12 specimens were prepared from paraffin-embedded tissue blocks. To minimize the risk of sampling errors and to increase the number of mast cells in the specimen, we opted for large cylinders (6 mm in diameter) of tissue.

7.2.3 DNA probes for FISH and antibodies for immunofluorescence

All DNA probes used in the current study are commercially available (Abbott Molecular Inc., Des Plaines, IL, USA). Numerical aberrations of chromosomes 8, and 9 were analyzed with spectrum orange (SO) labelled probes specific for the µ-satellite (centromeric) region mapping to 8p11.1-q11.1 and 9p11-q11, respectively. The mast cells were specifically detected by immunofluorescence staining using a primary monoclonal mouse anti-human mast cell tryptase antibody (clone AA1; DakoCytomation, Glostrup, Denmark) and a fluorescence-marked secondary goat anti-mouse antibody (Alexa Fluor 488; Invitrogen Corporation, Carlsbad, CA, USA).

7.2.4 Double staining: FISH and immunofluorescence

The tissue arrays were double-stained with a chromosome enumeration probe (CEP) specific for chromosomes 8 or 9 and a mast cell specific marker (monoclonal mouse anti-human mast cell tryptase antibody). Sections (6 µm thick) from the tissue array block were dewaxed, air-dried, and rehydrated in graded ethanol. After pretreatment according to the manufacturer's instructions, tissue sections were denatured at 73 °C in a 70 % formamide/2xSSC solution for 5 minutes.

Samples were then dehydrated in 70 %, 80 % and 100 % ethanol and subsequently treated with proteinase K (0.6 µg/ml, Sigma, St. Louis, MO) at 37 °C for 6 minutes. The hybridization mixtures consisted of 4 - 10 ng of each probe diluted in a hybridization mixture provided by the manufacturer. After hybridization at 37 °C in a humid chamber overnight with either CEP8 or CEP9 the slides were washed according to the manufacturer's instructions. Then the washed slides were again incubated overnight, but now with a primary monoclonal mouse anti-human mast cell tryptase antibody (DAKO), followed by a fluorescence-marked secondary goat anti-mouse antibody (ALEXA FLUOR 488, INVITROGEN) on the next day. The carefully washed slides were counterstained with DAPI II (Abbott Molecular Inc., Des Plaines, IL, USA) in an antifade-solution (125 ng/ml). As controls for probe specificity, standard chromosome preparations from peripheral blood lymphocytes were included in each hybridization procedure.

7.2.5 Scoring of FISH signals

At least 100 nuclei of tryptase-positive dermal mast cells were analyzed in different areas of the sections with a fluorescence microscope at 1000 times magnification. Only the cells showing clear morphologic features and positive staining for mast cell tryptase were scored. Mast cell tryptase positive cells with damaged nuclei were not counted. To avoid bias caused by sister chromatids of cells in S- or G2-phase, two signals were counted as one if they were situated very close (<0.05 µm) to each other. Slides were evaluated according to commonly accepted criteria [23-25]. The slides were only analyzed if two thirds of the keratinocytes and mast cells yielded interpretable signals. To avoid misinterpretation caused by inefficient hybridization, only cells with at least one bright centromere signal were counted. The proportion of cells with two or more signals and of cells with one signal was calculated for each patient. The definition of trisomy and monosomy was based on the results obtained in the nuclei of normal keratinocytes from these patients. In selected cases FISH analysis performed on different skin samples of the same patient yielded comparable results, thus indicating that the size of the samples (6 mm) was representative for the mast cell disorder.

8. Results

8.1 Controls

The definition of trisomy and monosomy was based on the results obtained in normal keratinocytes of the skin biopsies from our patients. These cells were chosen as internal controls because not enough mast cells were detected in sections of skin biopsies from normal controls to get reliable results.

The number and percentage of keratinocytes with monosomy, diploidy and trisomy are shown in Tab. 8-1and Tab. 8-2. In our samples the percentage of nuclei with three or more centromeric signals in the keratinocytes was 3.1 ± 2.2 % for chromosome 8 and 1.3 ± 1.0 % for chromosome 9. Unlike polysomic nuclei the proportion of nuclei with only one centromeric signal differed considerably between the tested chromosomes and was 24.3 ± 11.4 % and 31.4 ± 5.9 % for chromosome 8 and 9, respectively.

By definition, monosomy or trisomy are only diagnosed if the percentage of cells with none or one, and three or more signals, respectively, is higher than twice the mean of the signal counts of the keratinocytes added to two times the corresponding standard deviation [26, 27]. Based on these findings, the threshold values of trisomic cells for chromosome 8 and 9 were >10.6 % and >4.5 %, respectively. Furthermore, the threshold values for monosomy for chromosome 8 and 9 were >71.3 % and >74.7 %, respectively.

Tab. 8-1 Determination of limits for CEP8 by analysing keratinocytes

	Percentage of nuclei with		
case#	**monosomy**	**diploidy**	**trisomy**
1	7.0 %	84.0 %	9.0 %
3	20.0 %	78.0 %	2.0 %
3	14.0 %	84.0 %	2.0 %
3	12.0 %	86.0 %	2.0 %
5	25.0 %	73.0 %	2.0 %
6	10.0 %	88.0 %	2.0 %
10	44.0 %	53.0 %	3.0 %
10	21.0 %	73.0 %	6.0 %
12	34.0 %	63.0 %	3.0 %
13	29.0 %	68.0 %	3.0 %
14	48.0 %	51.0 %	1.0 %
15	28.0 %	70.0 %	2.0 %
17	24.0 %	70.0 %	6.0 %
20	18.0 %	78.0 %	4.0 %
22	23.0 %	75.0 %	2.0 %
27	31.0 %	68.0 %	1.0 %
Mean	24.3 %	72.6 %	3.1 %
SDEV	11.4 %	10.8 %	2.2 %
2*Mean + 2*SDEV	71.3 %		10.6 %

Tab. 8-2 Determination of limits for CEP9 by analysing keratinocytes

	Percentage of nuclei with		
case#	**monosomy**	**diploidy**	**trisomy**
3	30.1 %	68.9 %	1.0 %
5	37.0 %	62.0 %	1.0 %
8	32.8 %	67.2 %	0.0 %
12	36.0 %	63.0 %	1.0 %
14	19.3 %	78.9 %	1.8 %
15	38.9 %	59.5 %	1.6 %
24	29.2 %	69.0 %	1.8 %
27	27.4 %	69.4 %	3.2 %
28	32.1 %	67.9 %	0.0 %
Mean	31.4 %	67.3 %	1.3 %
SDEV	5.9 %	5.6 %	1.0 %
2*Mean + 2*SDEV	74.7 %		4.5 %

8.2 Numerical chromosomal aberrations in skin biopsies of patients with mastocytosis

A total of 16 patients were investigated with centromeric probes for chromosomes 8 and 9. Representative stainings are shown in Fig. 8-1a and Fig. 8-1b. In one case the signals for the chromosome 8 and in nine cases the signals for chromosome 9 were not interpretable. This was mainly due to loss of tissue, bad tissue quality and insufficient amount of material. Taken together numerical aberrations for chromosome 8 and 9 were found in 6 of 16 patients (38 %). The most frequent aberration was trisomy 9 (Tab. 8-3) which was detected in 5 of 7 cases (71 %). In contrast, trisomy 8 (Tab. 8-4). was only found in 1 of 15 cases (7 %). None of the patient showed trisomy for both chromosomes. Furthermore, none of the examined cases demonstrated a loss or a monosomy for chromosomes 8 or 9.

Tab. 8-3 Determination of trisomy 9 in mast cells

			Percentage of nuclei with		
case	**age**	**sex**	**monosomy9**	**diploidy9**	**trisomy9**
1	45	m	N/C!	N/C!	N/C!
3	75	m	N/C!	N/C!	N/C!
5	54	m	31.0 %	62.0 %	**7.0 %**
6	40	f	N/C!	N/C!	N/C!
8	49	f	N/C!	N/C!	N/C!
9	46	m	N/C!	N/C!	N/C!
10	53	f	27.8 %	61.1 %	**11.1 %**
13	35	m	49.0 %	49.0 %	2.0 %
16	54	m	N/C!	N/C!	N/C!
17	66	f	29.0 %	64.0 %	**7.0 %**
18	25	f	32.0 %	62.0 %	**6.0 %**
24	48	f	N/C!	N/C!	N/C!
26	13	f	N/C!	N/C!	N/C!
27	47	m	N/C!	N/C!	N/C!
35	2	m	29.0 %	66.0 %	**5.0 %**
39	53	f	28.6 %	71.4 %	0.0 %

bold cases that fulfill the criteria for monosomy (>74.7 %) or trisomy (>4.5 %)

N/C! not countable (because of loss of tissue, bad tissue quality or insufficient amount of material)

Tab. 8-4 Determination of trisomy 8 in mast cells

			Percentage of nuclei with		
case	**age**	**sex**	**monosomy8**	**diploidy8**	**trisomy8**
1	45	m	25.0 %	72.0 %	3.0 %
3	75	m	17.0 %	66.0 %	**17.0 %**
5	54	m	28.0 %	65.0 %	7.0 %
6	40	f	19.6 %	71.4 %	8.9 %
8	49	f	34.0 %	61.0 %	5.0 %
9	46	m	50.0 %	48.0 %	2.0 %
10	53	f	43.9 %	56.1 %	0.0 %
13	35	m	28.0 %	65.0 %	7.0 %
16	54	m	62.0 %	35.0 %	3.0 %
17	66	f	49.0 %	48.0 %	3.0 %
18	25	f	51.0 %	46.0 %	3.0 %
24	48	f	13.3 %	86.7 %	0.0 %
26	13	f	33.0 %	63.0 %	4.0 %
27	47	m	28.0 %	69.0 %	3.0 %
35	2	m	40.0 %	50.0 %	10.0 %
39	53	f	N/C!	N/C!	N/C!

bold cases that fulfill the criteria for monosomy (>71.3 %) or trisomy (>10.6 %)

N/C! not countable (because of loss of tissue, bad tissue quality or insufficient amount of material)

According to our definition of the cut-off levels for trisomy 8 and 9 there were statistically significant ($p < 0.05$) differences between the percentages of nuclei with three or more signals in the keratinocytes as compared to the mast cells in cases of mastocytosis with trisomy 8 or 9 as shown in Tab. 8-1and Tab. 8-2. For chromosome 9 there was even a statistically significant ($p < 0.05$) difference in the percentages of nuclei with three or more signals between the keratinocytes and the mast cells of the cases not reaching the arbitrary defined cut-off level for trisomy 9. The frequencies of mono- and polysomies, as defined by FISH studies, as well as a certain degree of genetic heterogeneity within a single tumor, may depend on the definition of the cut-off levels. In our series, no case harbored monosomy and trisomy of the same chromosome or trisomy of both chromosomes, thus probably reflecting real biological events. Mazzucchelli et al. previously reported that quantitative analysis of the results may lead to the detection of more subtile differences among different lesions [28].

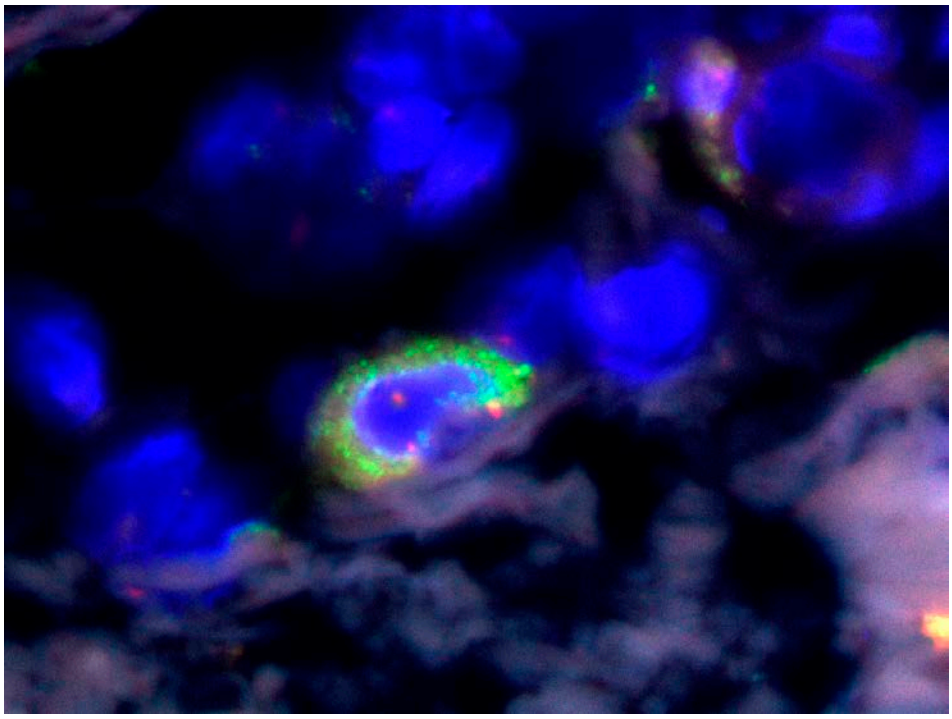

Fig. 8-1a Mast cell showing trisomy 8

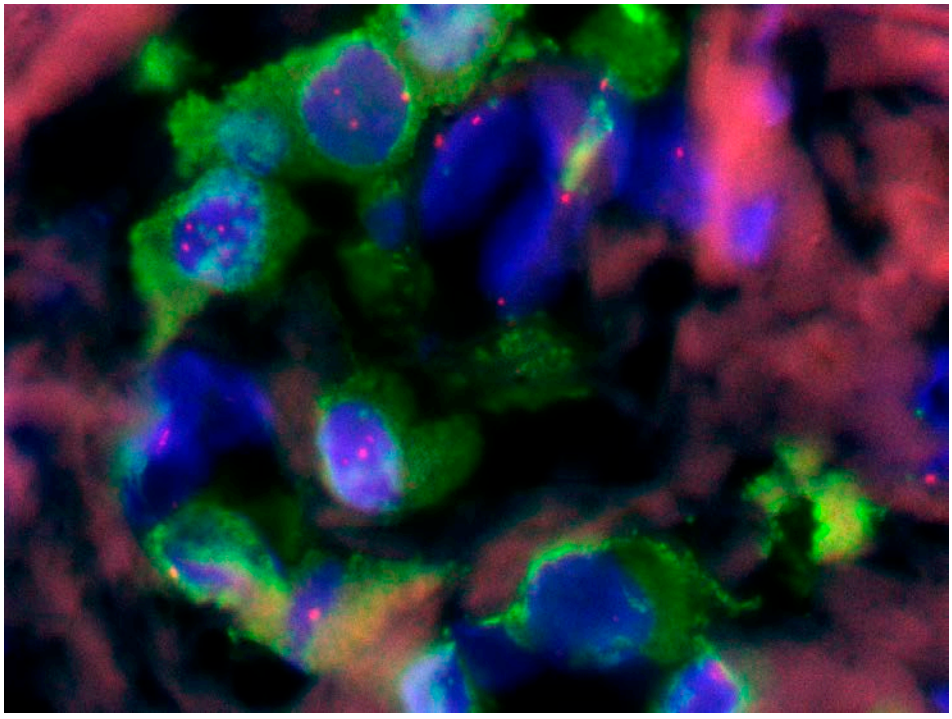

Fig. 8-1b Mast cells showing trisomy 9

Fig. 8-1a and Fig. 8-1b Mast cells are stained by immunofluorescence, therefore showing green colored cytoplasm. All nuclei are colored blue by FISH. Chromosomes 8 and 9 are made visible by FISH appearing as red spots. It is clearly visible that the mast cells show trisomies (three red dots) of chromosomes 8 or 9 in contrast to the other cells.

9. Discussion

This is the first interphase cytogenetic study performed by FISH on series of skin biopsies from patients with cutaneous mastocytosis, done in a “macro-array” format. Our results show, that chromosomal aberrations are frequently present in mast cells from skin biopsies of patients with cutaneous mastocytosis. In particular, trisomy 9 was found more frequently than trisomy 8 and only polysomies and no monosomies for both chromosomes 8 and 9 were observed. Furthermore, this study demonstrates the feasibility of the double-staining technique with FISH and a cell specific marker for the detection of numerical chromosomal aberrations in a specific cell population in paraffin-embedded tissue samples.

There are a few technical issues that need to be addressed first when performing FISH on tissue sections. As a drawback of this method, one has to mention that assessing losses of chromosomes in histological sections is hampered by high thresholds of 20 % in bone marrow sections and 60 % in liver sections [28]. In our study using skin biopsy specimen of cutaneous mastocytosis the thresholds were even higher, i.e. cut-off levels for monosomy for chromosome 8 and 9 were >71.3 % and >74.7 %, respectively. Previously, Mazzucchelli et al. [28] had speculated that this issue could also arise due to the cutting artifacts that have an increasing influence in larger cells. This has been observed for the larger hepatocytes in comparison to the smaller cells of the bone marrow and thus may also be relevant for the relatively large mast cells in the skin. In contrast to chromosomal losses this problem seems to be of lower impact for the detection of chromosome gains and structural imbalances. A further technical point to be mentioned is the duration of tissue fixation. Since 24 h of fixation is normally taken as a standard for routine purposes, we focused on this time in our study. Nevertheless, based on our own experience with tissues taken from surgical resection specimens of the liver after four days or more of fixation, FISH can be impaired (data not shown). Therefore, it is important to point out that over-fixation should be avoided.

A major finding in this study is that chromosomal aberrations may already be found in mastocytosis limited to the skin. Chromosomal aberrations in systemic mastocytosis have previously been reported by Lishner et al. [8]. In accordance with Lishner et al. we detected far more cases with trisomy of chromosome 9 than of chromosome 8. In the study of Lishner et al. the percentage of trisomy 8 and 9 was 17 % and 83 %, respectively. In our cases a percentage of 7 % for trisomy 8 and of 71 % for trisomy 9 was observed. The somewhat higher rates of Lishner et al. may in part be explained by various factors. Firstly, Lishner et al used different probes to detect trisomy of chromosome 8 and 9; secondly, they examined peripheral blood mononuclear cells and we specifically examined mast cells in skin biopsies; thirdly, in contrast to our study, Lishner et al. only examined cases with systemic mastocytosis.

In contrast to Lishner et al and our study, Swolin et al. was unable to detect any chromosomal aberrations in mastocytosis [9]. The discrepancy between the study of Swolin et al. on the one hand and Lishner's [8] and our study on the other hand may again be explained by the use of different cellular material, probes and /or stage of disease. Swolin et al. examined bone marrow cells, whereas Lishner et al. used peripheral blood mononuclear cells. However, none of these studies have mentioned a way to exclude other bone marrow or blood cells from the scoring of FISH signals. Only in our study a specific determination of trisomies in mast cells was performed by a double-staining technique. Moreover, Swolin et al. and Lishner et al. used identical probes for chromosome enumeration, ours differed from theirs. Interestingly, Swolin et al. had previously reported results of conventional cytogenetic studies in mastocytosis with trisomy 8 in 1 out of 34 patients (3 %). Previous studies had also stated that FISH was a more sensitive tool for the detection of chromosomal aberrations than the classical cytogenetic methods, e.g. as shown in the examination of neuroendocrine tumors of the lung [26], in tumors of the pancreas[27] and in systemic mastocytosis [8]. In fact, the likelihood of detecting a mutational spot is presumably much higher by FISH analysis done selectively on numerous neoplastic cells within a large tumour area than in LOH (Loss of heterozygosity) studies performed on DNA extracted from microdissected tissue. It is well known that gene alterations within neoplastic tissue occur as a cumulative process. New mutations may generate cell clones with a growth advantage over other cancerous cells. The sensitivity of the FISH method is dependent on the cut-off point. In order not to overestimate the level of trisomic cells, we have used the highest value +2 SD of the control group (keratinocytes) as the cut-off point. By using this conservative cut-off levels we ruled out an over-interpretation of the FISH scoring.

Swolin et al. [5] and Martens et al. [6] reported that the most frequently occuring numerical chromosomal aberrations in bone marrow cells are additional chromosomes 8 and 9. It is commonly believed that myeloproliferative diseases have a clonal origin in a multipotent stem cell that gives rise to granulocytes, erythrocytes, megakaryocytes, monocytes, and macrophage cells [29]. It has been shown that mast cells are replenished from CD34 multipotent hematopoietic progenitors in the presence of mast cell growth factors [30, 31]. The presence of similar numerical chromosomal aberrations in these stem cell diseases suggests that mastocytosis is a hematopietic stem cell disorder similar to myeloproliferative diseases [1, 32]. These chromosome abnormalities may represent primary or secondary events in the malignant transformation.

There are several oncogenes on chromosome 8, e.g. c-myc located at 8q24 or FGFR-1 at 8p11. c-myc belongs to a family of retrovirus-associated DNA sequences (myc) originally isolated from an avian myelocytomatosis virus. The protooncogene myc (c-myc) codes for a nuclear protein which is involved in nucleic acid metabolism and in mediating the cellular response to growth factors.

Fibroblast growth factor receptor 1 (FGFR-1) is a transmembrane receptor tyrosine kinase. An overexpression of both genes may lead to tumorgenicity.

Other oncogenes are located on chromosome 9, e.g. JAK2 at 9p24 or ABL at 9q34. JAK2 is a Janus kinase subtype that is involved in signaling from growth hormone receptors, prolactin receptors, and a variety of cytokine receptors such as erythropoietin receptors and interleukin receptors. Dysregulation of Janus kinase 2 due to genetic translocations have been associated with a variety of myeloproliferative disorders. ABL belongs to retrovirus-associated DNA sequences originally isolated from the Abelson murine leukemia virus (Ab-MuLV). The proto-oncogene abl (c-abl) codes for a protein that is a member of the tyrosine kinase family. It is activated by translocation to bcr on chromosome 22 in chronic myelogenous leukemia. The Philadelphia chromosome produces a fusion gene (bcr-abl) that gives rise to a constitutively activated abl tyrosine kinase. This kinase led to the discovery of several small-molecule inhibitors, imatinib being the first and most successful of these. However, in some patients abl kinase point mutations result in resistance against imatinib. Overcoming imatinib resistance represents one of the biggest challenges facing clinicians in the modern management of CML [33]. Imatinib is also successfully used in the treatment of some patients with mastocytosis [34].

In conclusion, our study shows for the first time that a double-staining technique using FISH and direct immunofluorescence staining with an anti-tryptase antibody is a sensitive and powerful method for the detection of numerical chromosomal aberrations specifically in mast cells in tissue sections. Using this method we demonstrate that numerical chromosomal aberrations (i.e. of chromosome 9) are already a frequent finding in cutaneous mastocytosis. Clearly, larger studies will be necessary to verify the pathogenic and clinical importance of these chromosomal abnormalities in mast cell diseases.

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11. References

1. Travis, W.D., et al., Systemic mast cell disease. Analysis of 58 cases and literature review. Medicine (Baltimore), 1988. 67(6): p. 345-68.
2. Braun-Falco, O., G. Plewig, and H.H. Wolff, *Mastozytosen*, in *Dermatologie & Venerologie*1995, Springer Verlag: Berlin. p. 1431-8.
3. Bolognia, J.L., J.L. Jorizzo, and R.P. Rapini, *Mastocytosis*, in *Dermatology*2008, Mosby Elsevier Verlag. p. 1845-53.
4. Swolin, B., S. Rodjer, and G. Roupe, *Cytogenetic studies and in vitro colony growth in patients with mastocytosis.* Blood, 1987. **70**(6): p. 1928-32.
5. Swolin, B., A. Weinfeld, and J. Westin, *A prospective long-term cytogenetic study in polycythemia vera in relation to treatment and clinical course.* Blood, 1988. **72**(2): p. 386-95.
6. Mertens, F., et al., *Karyotypic patterns in chronic myeloproliferative disorders: report on 74 cases and review of the literature.* Leukemia, 1991. **5**(3): p. 214-20.
7. Swolin, B., S. Rodjer, and G. Roupe, *Cytogenetic studies in patients with mastocytosis.* Cancer Genet Cytogenet, 2000. **120**(2): p. 131-5.
8. Lishner, M., et al., *Trisomies 9 and 8 detected by fluorescence in situ hybridization in patients with systemic mastocytosis.* J Allergy Clin Immunol, 1996. **98**(1): p. 199-204.
9. Swolin, B., et al., *Trisomies 8 and 9 not detected with fish in patients with mastocytosis.* Am J Hematol, 2002. **70**(4): p. 324-5.
10. Maintz, L. and N. Novak, *Histamine and histamine intolerance.* Am J Clin Nutr, 2007. **85**(5): p. 1185-96.
11. Fritsch, P., *Mastozytosen*, in *Dermatologie und Venerologie*2004, Springer-Verlag: Berlin. p. 676-80.
12. Horny, H.P., K. Sotlar, and P. Valent, *Mastocytosis: state of the art.* Pathobiology, 2007. **74**(2): p. 121-32.
13. Valent, P., et al., *Diagnostic criteria and classification of mastocytosis: a consensus proposal.* Leuk Res, 2001. **25**(7): p. 603-25.
14. Horny, H.P. and P. Valent, *Diagnosis of mastocytosis: general histopathological aspects, morphological criteria, and immunohistochemical findings.* Leuk Res, 2001. **25**(7): p. 543-51.
15. Sperr, W.R., et al., *Clinical and biologic diversity of leukemias occurring in patients with mastocytosis.* Leuk Lymphoma, 2000. **37**(5-6): p. 473-86.
16. Casassus, P., et al., *Treatment of adult systemic mastocytosis with interferon-alpha: results of a multicentre phase II trial on 20 patients.* Br J Haematol, 2002. **119**(4): p. 1090-7.
17. Butterfield, J.H., *Response of severe systemic mastocytosis to interferon alpha.* Br J Dermatol, 1998. **138**(3): p. 489-95.
18. Hauswirth, A.W., et al., *Response to therapy with interferon alpha-2b and prednisolone in aggressive systemic mastocytosis: report of five cases and review of the literature.* Leuk Res, 2004. **28**(3): p. 249-57.
19. Petty, H.R., *Fluorescence microscopy: established and emerging methods, experimental strategies, and applications in immunology.* Microsc Res Tech, 2007. **70**: p. 687-709.
20. Gall, J.G. and M.L. Pardue, *Formation and detection of RNA-DNA hybrid molecules in cytological preparations.* Proc Natl Acad Sci U S A, 1969. **63**(2): p. 378-83.

21. Manning, J.E., et al., *A new method of in situ hybridization.* Chromosoma, 1975. **53**(2): p. 107-17.
22. Johnson, K.L., et al., *Chromosome aberrations of clonal origin in irradiated and unexposed individuals: assessment and implications.* Radiat Res, 1999. **152**(1): p. 1-5.
23. Hopman, A.H., et al., *Detection of numerical chromosome aberrations using in situ hybridization in paraffin sections of routinely processed bladder cancers.* Mod Pathol, 1991. **4**(4): p. 503-13.
24. Sauter, G., et al., *Physical deletion of the p53 gene in bladder cancer. Detection by fluorescence in situ hybridization.* Am J Pathol, 1994. **144**(4): p. 756-66.
25. Sauter, G., et al., *Fluorescence in situ hybridization for detecting erbB-2 amplification in breast tumor fine needle aspiration biopsies.* Acta Cytol, 1996. **40**(2): p. 164-73.
26. Gugger, M., et al., *Quantitative expansion of structural genomic alterations in the spectrum of neuroendocrine lung carcinomas.* J Pathol, 2002. **196**(4): p. 408-15.
27. Soldini, D., et al., *Progressive genomic alterations in intraductal papillary mucinous tumours of the pancreas and morphologically similar lesions of the pancreatic ducts.* J Pathol, 2003. **199**(4): p. 453-61.
28. Mazzucchelli, L., et al., *Interphase cytogenetics in oncocytic adenomas and carcinomas of the thyroid gland.* Hum Pathol, 2000. **31**(7): p. 854-9.
29. Strife, A. and B. Clarkson, *Biology of chronic myelogenous leukemia: is discordant maturation the primary defect?* Semin Hematol, 1988. **25**(1): p. 1-19.
30. Kirshenbaum, A.S., et al., *Demonstration of the origin of human mast cells from CD34+ bone marrow progenitor cells.* J Immunol, 1991. **146**(5): p. 1410-5.
31. Agis, H., et al., *Monocytes do not make mast cells when cultured in the presence of SCF. Characterization of the circulating mast cell progenitor as a c-kit+, CD34+, Ly-, CD14-, CD17-, colony-forming cell.* J Immunol, 1993. **151**(8): p. 4221-7.
32. Horny, H.P., et al., *Blood findings in generalized mastocytosis: evidence of frequent simultaneous occurrence of myeloproliferative disorders.* Br J Haematol, 1990. **76**(2): p. 186-93.
33. Swords, R., Y. Alvarado, and F. Giles, *Novel Abl kinase inhibitors in chronic myeloid leukemia in blastic phase and Philadelphia chromosome-positive acute lymphoblastic leukemia.* Clin Lymphoma Myeloma, 2007. **7 Suppl 3**: p. S113-9.
34. Hoffmann, K.M., et al., *Successful treatment of progressive cutaneous mastocytosis with imatinib in a 2-year-old boy carrying a somatic KIT mutation.* Blood, 2008. **112**(5): p. 1655-7.