PROGNOSTIC FACTORS IN PRIMARY

MERKEL CELL CARCINOMA

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Academic dissertation

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To my family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals. Some unpublished data are also presented.

I Koljonen, V., Böhling T., Granroth G., Tukiainen E.: Merkel cell carcinoma: A clinicopathological study of 34 patients *European Journal of Surgical Oncology*, 2003. **29**(7): 607-610

II Larramendy M., Koljonen V., Böhling T., Tukiainen E., Knuutila S.: Recurrent DNA copy number changes revealed by comparative genomic hybridisation in primary Merkel cell carcinomas *Modern Pathology*, 2004. **17**(5): 561-567

III Koljonen, V., Tukiainen E., Haglund C., and Böhling T.: Expression of cyclin A in Merkel cell carcinoma *APMIS*, 2004. **112**(1): 39-44

IV Koljonen V., Lassus P., Tukiainen E., Ristimäki A., Haglund C and Böhling T.: Cyclooxygenase-2 Expression in Primary Merkel Cell Carcinoma *Journal of Cutaneous Pathology (in press)*

V Koljonen V., Jahkola T., Tukiainen E., Haglund C and Böhling T.: Tenascin-C in primary Merkel cell carcinoma, *Journal of Clinical Pathology (in press)*

ABBREVIATIONS

CDK cyclin-dependent kinases

CDKI cyclin-dependent kinase inhibitors

CGH comparative genomic hybridisation

CK-20 cytokeratin 20

CrA chromogranin A

ECM extracellular matrix

EGF epidermal growth factor

EM electron microscopy

FHIT fragile histidine triad

HE haematoxylin-eosin

LI labelling index

LOH loss of heterozygosity

MAP microtubule-associated protein

MC Merkel cell

MCC Merkel cell carcinoma

NSAIDS non-steroidal anti-inflammatory drugs

NSE neuron-specific enolase

OS overall survival

SCLC small cell lung carcinoma

SEER Surveillance, Epidemiology, and End Results Program

Skp2 S-phase kinase-associated protein 2

SLNB sentinel lymphnode biopsy

TEM transmission electron microscopy

Tn-C tenascin-C

TTF-1 thyroid transcriptor factor 1

ABSTRACT

Introduction: Merkel cell carcinoma (MCC) is an unusual primary neuroendocrine carcinoma of the skin. The aggressive course of the disease shows rapid progression of the primary tumour, early and frequent lymph nodebasin metastases and common systemic metastasis. MCC mainly affects sun-exposed areas of elderly persons. Half of the tumours are located in the head and neck region. Depending on the length of the follow-up, up to 40% of patients have local recurrences; over 50% develop lymph node metastasis and 36% systemic metastasis. The recommended initial treatment is extensive local excision. Little is known about the biology and prognostic factors of MCC. The aim of this study, therefore, was to investigate both the clinical and molecular features of this tumour and, further, to compare the molecular changes with the clinical outcome.

Materials and Methods: In the search for biological, tumour-related and patient-related prognostic factors for this carcinoma, altogether 38 MCC samples were assessed with the aid of complete clinical data. The clinicopathological data of the patients were analysed to establish the factors influencing survival. Immunohistochemical analyses for cyclin-A, Cox-2 and tenascin-C (Tn-C) were performed. The results were compared with the clinical outcome and submitted to statistical analysis. In addition, chromosomal aberrations in the primary MCC samples were investigated by comparative genomic hybridisation and compared with clinical data.

Results: The overall survival rate was largely influenced by tumour size: the larger the tumour the greater the negative impact on survival (tumour size ≥ 2 cm $p < 0.009$). Only one clinically favourable prognostic factor was established, i.e., success in reconstructing the defect with a split thickness skin graft or local flap ($p < 0.02$). Tumours expanding to metastasis expressed cyclin-A, Cox-2 and Tn-C more extensively than did tumours with a more benign course of disease. DNA copy number changes were mainly seen in large tumours. The tumours with detected changes expanded to metastasis three times as frequently as did tumours in which no chromosome aberrations could be demonstrated.

Conclusions: The highly malignant nature of MCC was confirmed in this series of thirty-eight patients. Large tumour size indicated poor survival, and therefore aggressive surgical treatment is strongly advocated. On the molecular level, MCCs exhibit multiple DNA copy number changes at many chromosomal locations. Gains in chromosome 6 seemed to be especially typical of large tumours. Tumours with DNA copy number changes had a higher tendency for metastasis. The prognosis tended to be poorer in the event of overexpression of cyclin-A and Cox-2. However, Cox-2 expression levels were usually low, indicating that this molecule is not a target for treatments. Tn-C expression correlated with large tumour size and consequently poor prognosis.

INTRODUCTION

Merkel cell carcinoma (MCC) is a frequently fatal disease, and patients have a poor chance of survival (Boyle et al. 1995). Moreover, MCC lacks distinguishing clinical features, and thus by the time the diagnosis is made, the tumour will usually have expanded to metastasis.
MCC was first described in 1972 (Toker 1972). Since then, more than 600 cases have been reported, the majority in small series of patients. Over half of the tumours have been located in the head and neck region. Most of the reports concern single cases or epidemiological studies. The epidemiological studies have revealed that large tumour size, male sex, truncal site, nodal/ distant disease at presentation, and duration of disease before presentation, are poor prognostic factors (Hitchcock et al. 1988; Medina-Franco et al. 2001; Agelli and Clegg 2003). Thus far, no biological prognostic marker has been discovered for MCC although many have been tested.

In an effort to understand the behaviour of a particular tumour, one must employ several methods to explore the malignant processes. Epidemiological studies provide information on the characteristic course of the disease but not on the tumour itself. In order to study the biology of the tumour one must penetrate deeper, to the molecular level and unravel the changes in cell physiology (Hanahan and Weinberg 2000). These include regulators of and contributors to growth, invasion and metastasis. In some malignant tumours, such changes have proved to be of prognostic significance.

Prognostic factors can be grouped into patient-related, tumour-related and treatment-related categories (Altwein and Luboldt 1999). In the search for prognostic markers in MCC, these three categories were examined in an effort to broaden the understanding of the natural course of MCC. This was done by gathering ample numbers of specimens and carefully recording each patient's disease history. The impact of immunohistochemical findings on survival was tested statistically.

Every unexpected event in the course of the disease (recurrence of primary tumour, metastatic dissemination succeeding surgery, and oncological treatments) diminishes the patient's quality of life. Our aim, therefore, was to produce information, and easy-to-measure, repeatable prognostic factors that would help pinpoint the right treatment protocols for patients and thus eliminate unnecessary procedures.

REVIEW OF THE LITERATURE

1. The Merkel cell

1.1 History

The Merkel cell (MC) was first described by the German histopathologist Friedrich Sigmund Merkel in his classic article published in 1875: Merkel F: “Tastzellen und Tastkörperchen bei den Haustieren und beim Menschen”, (Arkiv für Mikroskopische Anatomie und Entwicklungsmechanik 11: 636-652). He demonstrated the existence of touch cells in the snout skin of pigs, calling them “Tastzellen” because of their putative function in the touch sensation. These clear-staining cells at the dermoepidermal junction were in close proximity to myelinated nerve fibres. Merkel postulated that the cells acted as mechanoreceptors in all animals.

1.2 Distribution of Merkel cells

MCs are normal constituents of the basal layer of the epidermis and the follicular epithelium (Briggaman and Wheeler 1975). They are scarce in normal skin, but are present in high numbers and form clusters in areas of sensory perception, such as fingertips, the tip of the nose, tactile hair follicles, the lips, the proximal nail fold and the dorsum of the feet. They usually occur as single cells in the basal layer of the epidermis. In close association with primary nerve endings in the skin, they form the MC-axon complex. There are more MCs in sun-exposed than in non-sun-exposed areas of the skin (Moll et al. 1990a). Chronic sun exposure results in MC hyperplasia (Gould et al. 1985; Hartschuh and Schulz 1997). Kanitakis and co-workers have proposed that MC hyperplasia is unrelated to epidermal proliferation but rather that it is specific to a limited number of skin diseases such as actinic keratosis, fibrous papules of the face, and conditions with immature hair follicle differentiation (Kanitakis et al. 1998).

1.3 Function in developing embryo and adult

In human development, MCs can be detected in the epidermis in the 8th gestational week (Moll et al. 1996). In the developing embryo, MCs are postulated to be involved in the formation of the subepidermal nerve plexus (Vos et al. 1991; Narisawa et al. 1992), and in the formation and proliferation of eccrine sweat glands and hair follicles (Kim and Holbrook 1995). In the adult MCs are thought to act as slow acting type-I mechanoreceptors. Together with sensory nerve endings, they form MC-axon complexes that are activated by steady skin indentation (Ogawa 1996). The function of MCs in this complex is, however, enigmatic. Two possible functions have been proposed: either they may act as attracters of developing or regenerating type I nerve fibres (He et al. 1999) or they may have neuromodulatory or neuroregulatory functions in the basal

epidermis, such as keratinocyte proliferation stimulation, maintenance of the differentiation of keratinocytes, or the release of bioactive substances to subepidermal structures (Tachibana 1995). After denervation, MCs degenerate and are reduced in number. They do not, however, disappear completely (English 1977), continuing to survive in transplanted fasciocutaneous and musculocutaneous flaps. Their survival seems to correlate with recovery of the touch sensation (Vesper et al. 2003).

1.4 Origin of the Merkel cell

The origin of the MC is still controversial. The cell may derive either from the epithelial cells of the epidermis or from the neural crest migrating to the epidermis during embryogenesis. In the 1980s and 1990s, the epidermal origin from keratinocytes with an aberrant differentiation (Heim and Mitelman 1995) was the prevailing hypothesis (Frigerio et al. 1983; Moll et al. 1986a; Compton et al. 1990; Moll et al. 1996), as suggested by the epidermal location of the MC, the expression of cytokeratins and the results of skin transplantation experiments (Lyne and Hollis 1971; English et al. 1980). Recent studies, however, have provided strong evidence in support of the neural crest origin (Grim and Halata 2000; Szeder et al. 2003), as determined by the ontogenetic origin of MCs in Wnt1-cre/R26R compound transgenic mice, in which neural crest cells are marked indelibly (Szeder et al. 2003).

1.5 Structure

On the light microscopy level, MCs are large, oval, amphophilic, clear cells situated in the basal or suprabasal layer of the epidermis. They are not easily distinguished from other non-keratinocytic epidermal cells, e.g. melanocytes and Langerhans cells, by light microscopic immunohistochemistry. Special techniques such as immunohistochemistry, electron microscopy (EM) or transmission electron microscopy (TEM) are therefore required for their identification. MCs express cytokeratin-20 (CK-20) (Moll et al. 1995) and are furthermore exclusively cromogranin-A (CrA) immunoreactive (Hartschuh et al. 1989). EM reveals cytoplasmic dense-core granules (Warner et al. 1983). TEM has shown that the cell has microvilli radiating from the cell body and secretory granules (Munger 1965; Takahashi-Iwanaga and Abe 2001). Toyoshima and co-workers have demonstrated the presence of microvilli by immunohistochemistry, even by light microscopy (Toyoshima et al. 1998).

2. Merkel cell carcinoma

The Merkel cell carcinoma (MCC) was first described in 1972, when Toker presented the first five cases under the name “trabecular carcinoma of the skin”, assuming them to represent an eccrine, sweat gland-derived carcinoma (Toker 1972). In electron microscopic studies, Tang and Toker later identified dense-core neuroendocrine granules within the tumour cells, thus demonstrating their origin from MCs (Tang and Toker 1978).
The “cell of origin” of MCC is, however, still speculative. There are morphological and biological similarities between the MC and MCC. The common presence of neuroendocrine granules (Miettinen et al. 1983) and the positive immunostaining for cytokeratin-20 (CK-20), for instance, provide evidence for MC origin (Sadahira et al. 1987), although this may indicate differentiation rather than origin. However, certain differences also exist between the MC and MCC, such as the fibrous whorls and neurofilaments that are seen in MCC but not in normal MCs. Another argument against the MC as the cell of origin for MCC is that mitoses have not been detected in human MCs (Vaigot et al. 1987; Moll et al. 1996). There is also a contradiction between the location of the cell in the skin in MCs and in MCC. Moreover, the tumour practically always involves the dermis, sparing the epidermal structures.

2.1 Synonyms

Toker called the first five cases of MCC “trabecular carcinoma of the skin”(Toker 1972). This name derived from the trabecular architecture of the tumour, which is the most characteristic, but also the most uncommon, configuration of three histological patterns (see 2.3 Histology). Over the years, the name of this tumour has been the subject of lively discussion. During the past three decades, several names and synonyms have been proposed; either based on histological features or derived from the term “Merkel cell”. These include derivatives such as “Malignant Merkel-cell tumour” (Rywlin 1982), “Merkel cell tumour” (Fetissof et al. 1983) or “Merkel cell tumour of the skin “ (Cremer and Totovic 1983). It was not until the mid 1980s that the term “Merkel cell carcinoma” was established. In the year 1980, Johannessen and co-workers seem to have used this term for the first time (Johannessen and Gould 1980). The term “Primary neuroendocrine carcinoma of the skin” was coined to reflect the pathophysiology of the disease. Other interesting synonyms from the literature are presented in Table 1.

2.2 Small round blue cell tumours

The histology of MCC is typical of small round blue cell tumours, an entity that includes a wide variety of highly malignant tumours: the Ewing family of tumours, olfactory neuroblastoma (esthesioblastoma), rhabdomyosarcoma, neuroblastoma, lymphoma, desmoplastic small cell tumour, osteosarcoma, small cell lung carcinoma (SCLC), small cell melanoma and

mesenchymal chondrosarcoma (Tarkkanen and Knuutila 2002; Pisick et al. 2003). MCC shares some common histological, clinical features with SCLS, a primary neuroendocrine carcinoma of the lung (Gould et al. 1985;Meeuwissen et al. 1995).

2.3 Histology

Diagnosis is based on typical histology representation on haematoxylin-eosin (HE) -stained slides together with the results of immunohistochemistry (Johansson et al. 1990). A typical histological finding in MCC is the presence of tumour tissue within the dermis with repeated extensions to underlying subcutaneous tissue. The epidermis, papillary dermis and adnexal structures are not usually involved. Cytological features include sparse cytoplasm with uniform, monotonous medium-sized nuclei and abundant mitoses (Warner et al. 1983). Sometimes trabeculae and pseudorosettes are seen. All these cytomorphological features can be present as well in fine needle aspirates (Skoog et al. 1990). EM studies show that the ultrastructure of neuroendocrine granules of the tumour are similar to that of normal MCs. The tumour cells are round to ovoid and are intimately apposed to other tumour cells. The most consistent findings are the aggregation of intermediate filaments in a paranuclear location and the existence of membrane-bound dense core granules. The granules are usually concentrated in the periphery or in dendrite-like processes (Moll et al. 1986b). Only 10 % of all MCC tumours are intraepidermal (Brown et al. 2000). Sometimes the tumour can spread in a pagetoid manner (Leboit et al. 1992; Hashimoto et al. 1998).

Histologically, MCC can be classified into three distinct subtypes (Gould et al. 1985; Ratner et al. 1993; Haag et al. 1995). This classification is usually reserved for the histological studies. The first of these is the **trabecular subtype,** described originally by Toker (Toker 1972). This is the least frequent histological pattern. Cells are arranged in distinctly organoid clusters and trabeculae with occasional ribbons. Individual cells are round to polygonal in shape and are compactly arranged. The tumour cell cytoplasm is comparatively abundant and often well defined. Mitoses are few to moderate in number. This type of tumour usually occurs adjacent to adnexal structures, particularly hair follicles. The **intermediate subtype** is the most frequent histological subtype (Johansson et al. 1990). It exhibits a solid and diffuse growth pattern. Cells are less compactly arranged, and the cytoplasm is less abundant than in the trabecular type. Mitoses and focal areas of necrosis are frequent. These tumours usually arise adjacent to adnexa, but may invade the epidermis. The clinical behaviour is more aggressive than that of tumours of the trabecular type. The **small cell type** mimics small cell tumours of other sites, e.g. SCLC (Schmidt et al. 1998). The tumours arise in the dermis and appear as solid sheets and clusters of cells. Areas of necrosis and “crushing” artefacts are frequent. The clinical behaviour of this subtype appears to be as aggressive as that of the intermediate subtype.

Table 1 MCC synonyms used in the literature over the years

Trabecular carcinoma of skin; Toker C. Trabecular carcinoma of skin. Arch Derm 1972; 105:107 - 110

Cutaneous trabecular carcinoma; Abaci IF et al. Multicentric amyloid containing cutaneous trabecular carcinoma: case report with ultrastructural study. J Cutan Pathol 1979;6:292 - 303

Primary small cell carcinoma of skin; Taxy JB et al. Primary Small Cell Carcinoma of the skin. Cancer 1980; 46:2308 - 2311

Neuroendocrine carcinoma of skin; Gould VE et al. Neuroendocrine carcinomas of the skin: light microscopic, ultrastructural and immunohistochemical analysis. Ultrastruct Pathol 1980; 1; 499 - 509

Merkel cell neoplasm; Johannesen JV et al. Neuroendocrine Carcinoma associated with calcitonin production: a Merkel Cell Carcinoma? Human Pathol 1980; 11(suppl): 586 - 589

Cutaneous APUDoma; DeWolf-Peeters C et al. A cutaneous APUDoma or Merkel cell carcinoma? A morphologically recognizable tumour with a biological and histological malignant aspect in contrast with its clinical behaviour. Cancer 1980:46:1810 - 1816

Murky cell carcinoma;Stern JB "Murky" cell carcinoma (formerly trabecular carcinoma) Am J Dermapathol 1982:4:509 – 11

Malignant Merkel cell tumour; Rywlin AM: Malignant Merkel-cell tumour is a more accurate description than trabecular carcinoma Am J Dermapathol 1982:4:513 - 5

Small-cell neuroepithelial tumour of skin; Pollack SV et al. Small-cell neuroepithelial tumour of skin: a Merkel cell Carcinoma? J Dermal Surg Oncol 19828116 - 122

Merkel cell tumour; Fetissof, F., B. Arbeille-Brassart, et al. (1983). "[Merkel cell tumour]." Ann Pathol 3(4): 285-91

Merkel cell tumour of the skin; Cremer, H. and V. Totovic (1983). "[Merkel cell tumour of the skin. Light and electron microscopic study of 5 cases]." Pathologe 4(6): 287-93

Endocrine carcinoma of the skin; Goepfert H et al. Merkel cell carcinoma (endocrine carcinoma of the skin) of the head and neck Arch Otolaryng 1984; 110:707 - 12

Merkeloma; Schmidt-Winterscheidt, M. and H. Oberste-Lehn (1985). "[Merkeloma (Merkel cell carcinoma) from the clinical viewpoint based on 4 cases]." Z Hautkr 60(15): 1187-90, 1195-7

Small cell carcinoma of skin;Cullen, K. W., S. G. Subbuswamy, et al. (1985). "Small cell carcinoma of skin: a report of two cases." Br J Plast Surg 38(4): 575-8

Cutaneous merkeloma;Buffa, R., G. Rindi, et al. (1987). "Synaptophysin immunoreactivity and small clear vesicles in neuroendocrine cells and related tumours." Mol Cell Probes 1(4): 367-81

Epidermotropic primary neuroendocrine carcinoma of the skin;Rocamora A et al. Epidermotropic primary neuroendocrine (Merkel cell) carcinoma of the skin with Pautrier-like microabscesses J Am Acad Dermatol 1987:16:11163 - 8

Anemone cell tumour; Wills EJ Anemone cell tumour with neuroendocrine differentiation (presumed Merkel cell carcinoma) Ultrastruct Pathol 1990;14:161 - 171

Cutaneous small cell undifferentiated carcinoma (CSCUC); Datta, C. K. and C. B. Mendoza, Jr. (1999). "Merkel cell carcinoma: an aggressive neoplasm." W V Med J 95(3): 127-9

2.4 Occasional differentiation

There have been some sporadic reports of differentiation towards biphasic representation such as leiomyosarcomatous differentiation in primary tumours and rhabdomyosarcomatous differentiation in lymph node metastasis (Cooper et al. 2000; Fernandez-Figueras et al. 2002). Sometimes the presence of squamous or eccrine differentiation is prominent (Gould et al. 1988; Smith and Patterson 2001).

2.5 Immunohistochemistry - differentiation

The tumour usually expresses both epithelial and neuroendocrine markers, and thus exhibits both epithelial and neuroendocrine differentiation.

2.5.1 Epithelial differentiation

Of the intermediate filaments, MCC expresses low-molecular-weight cytokeratins (keratins 8,18,19 and 20), the simple epithelial type being the most marked (Miettinen et al. 1983; Moll et al. 1986b). The most important keratin in differential diagnostics is cytokeratin-20 (CK-20). CK-20 is a low-molecular-weight cytokeratin, that was originally identified by Moll and co-workers in two-dimensional gel electrophoresis of cytoskeletal extracts of intestinal epithelia (Moll and Franke 1985; Moll et al. 1990b). In normal tissues, only the gastrointestinal epithelium, urothelium, and MCs express CK-20 (Miettinen 1995). Recently it has been used for determining the site of origin in adenocarcinomas of uncertain origin (Loy and Calaluce 1994). Gastrointestinal adenocarcinomas are frequently CK-20 positive, whereas adenocarcinomas of the lung or female genital tract (with the exception of mucinous adenocarcinoma of the ovary) are usually CK-20 negative.

In the skin, the epidermis and skin appendages are regularly CK-20 negative. Rare isolated cells in the basal layer of the epidermis and external root of hair follicles stain positively for CK-20, representing normal MCs (Chan et al. 1997). Considered a sensitive and specific marker for MCC, CK-20 is helpful in efforts to distinguish between MCC and other malignant neoplasms, since it is not expressed in neuroendocrine carcinomas of other sites, such as SCLC (Moll et al. 1992; Scott and Helm 1999). In some 5 –25% of MCC cases, CK-20 is negative (Chan et al. 1997; Barrett et al. 2000).

Tissue-specific transcription factors control cell determination and differentiation. TTF-1 is a tissue specific transcription factor expressed in epithelial cells of the thyroid and lung (Lazzaro et al. 1991). TTF-1 is useful in distinguishing primary pulmonary adenocarcinoma from metastatatic carcinomas, identifying differentiated thyroid neoplasms, distinguishing mesothe-

lioma from pulmonary adenocarcinoma, and distinguishing SCLC from MCC (Ordonez 2000;Byrd-Gloster et al. 2000; Cheuk et al. 2001).
Combining TTF-1 with CK-20 provides a sound basis for diagnosis. Most adenocarcinomas from other sites (breast, lung, endometrium) and neuroendocrine carcinomas such as SCLC are essentially negative for CK-20 (Moll et al. 1992). MCC is negative for S-100 protein, a widely used marker of malignant melanoma cells (Cheuk et al. 2001; Taira et al. 2002), and for leukocyte-common antigen, excluding cutaneous lymphomas (Battifora and Silva 1986). Table 2 presents the immunohistochemistry for the differentiation diagnosis of MCC.

Table 2 Immunohistochemical markers in the differential diagnosis of MCC. SCLC small cell lung carcinoma, MM malignant melanoma, LGNEC low grade neuroendocrine carcinomas. CK-20 cytokeratin 20, TTF-1 thyroid transcription factor 1, NSE neuron-specific enolase, Cr-A cromogranin-A, NFP neurofilament proteins, CD56 neural adhesion molecule, MAP-2 microtubule associated protein-2, LCA leukocyte common antigen, SYP synaptophysin, + positive, +/- mostly positive, -/+ mostly negative, - negative

	CK-20	**TTF-1**	**NSE**	**S-100**	**Cr-A**	**SYP**	**NFP**	**CD56**	**MAP-2**	**LCA**
MCC	+	-	+	-	+/-	+/-	+	+	+	-
SCLC	-	+	+/-	-	-/+	+	-/+	+	+	-
MM	-	-	-	+	-		-		+	-
LGNEC	+		+		+	+	-/+			
Malignant lymphoma	-	-	-	-	-		-/+			+

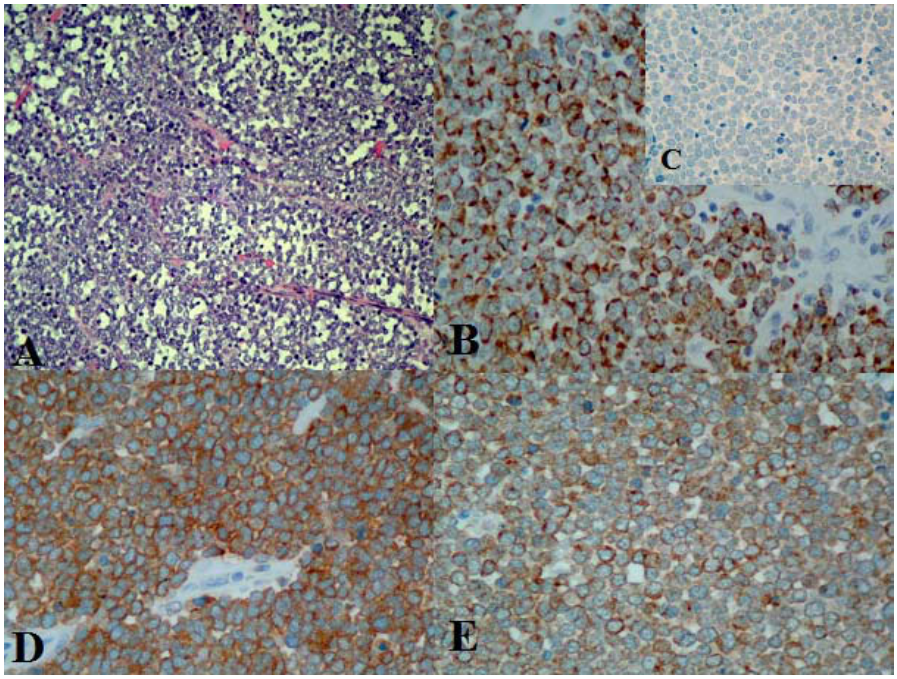
2.5.2 Neuroendocrine differentiation

Due to neuroendocrine origin, MCC always stains positively for neuron-specific enolase (NSE), which is a general marker of neuroendocrine tumours (Sibley and Dahl 1985; Leong et al. 1986; Metz et al. 1998). CD56, or neural cell adhesion molecule (NCAM), has recently been demonstrated to be a neuroendocrine marker of the pulmonary neuroendocrine cell system as well as MCC (Gallego et al. 1995; Kurokawa et al. 2003). Chromogranin A (CrA), a member of the chromogranin family, is a major protein that accounts for a large amount of the

soluble matrix of neurosecretory granules. These granules are present throughout the neuroendocrine system and in neurons. First isolated from chromaffin cells of the adrenal medulla (Banks and Helle 1965; Helle 1966), CrA is the most widely distributed marker of endocrine tumours (Weiler et al. 1988). MCC shows a focal, positive immunoreaction to CrA (Wilson and Lloyd 1984; Haneke et al. 1993), Figure 1. Synaptophysin is a transmembrane channel protein of small presynaptic vesicles. It is expressed in neuroendocrine and neural cells and diffusely in neuroendocrine system cells (Wiedenmann et al. 1986; Wiedenmann and Huttner 1989). Both primary and metastatic neuroendocrine carcinomas are habitually synaptophysin positive (Gould et al. 1987; Miettinen 1987). MCC consistently shows positive immunoreactions to synaptophysin (Buffa et al. 1987), Figure 1.

New markers of neuroendocrine differentiation introduced for MCC are the microtubule-associated proteins (MAPs). These are the major component of the cytoskeleton family of proteins associated with the microtubule assembly of the central and peripheral nervous system (Liu et al. 2001; Liu et al. 2003b). Cytoskeleton functions include the regulation of cell shape and polarity during differentiation and proliferation. They can modulate intracellular transport, surface receptors, mitosis and cell motility (Avila 1992; Maccioni and Cambiazo 1995). Specific MAPs have been identified in specific cell types (Maccioni and Cambiazo 1995). MAP-2, for instance, is a highly sensitive and specific marker of neuroendocrine differentiation (Fang et al. 2001; Liu et al. 2001; Liu et al. 2003b). Liu and co-workers demonstrated MAP-2 expression in all MCC samples, even though CK-20 staining was negative (Liu et al. 2003a). Various neuropeptides can be detected in MCC by immunohistochemistry. Among them are vasoactive intestinal peptide (VIP), calcitonin, adrenocorticotropic hormone (ACTH) (Leong et al. 1986) and substance P (Silva et al. 1984b). However, these lack clinical significance and have only been demonstrated in limited series.

Figure 1 Immunohistochemical staining of primary MCC for differential diagnosis and neuroendocrine differentiation. **A** HE staining, the tumour cells have round nuclei, original magnification 200x. **B** Positive CK-20 staining, showing typical punctate pattern of immunostaining, original magnification 400x. **C** Negative staining for TTF-1, original magnification 400x. **D** Cr-A staining showing a positive red staining reaction in the tumour cells, original magnification 400x and **E** Positive SYP staining, original magnification 400x.



2.6 The clinical picture of MCC

2.6.1 Clinical presentation and staging

The clinical presentation of this tumour is rather non-specific. MCC is usually, at least at early stages of the disease, defined as a small painless erythematous intradermal mass, usually with no ulceration. (Hitchcock et al. 1988; Yiengpruksawan et al. 1991) Especially small tumours in particular can appear somewhat benign, whereas large tumours have an unquestionably malignant appearance. Because of the rare nature of this neoplasm, it is often mistaken for more common skin tumours of epithelial origin. The largest may even resemble sarcomas. Figure 2.

There is no classification scheme for MCC such as there is for most carcinomas or sarcomas. At present, staging of most malignant tumours is based on the T(umour) N(odes) M(etastasis) classification (TNM classification of malignant tumours - 6th edition, 2002). Conserning the skin malignancies the TNM classification applies to carcinomas of the skin in general and malignant

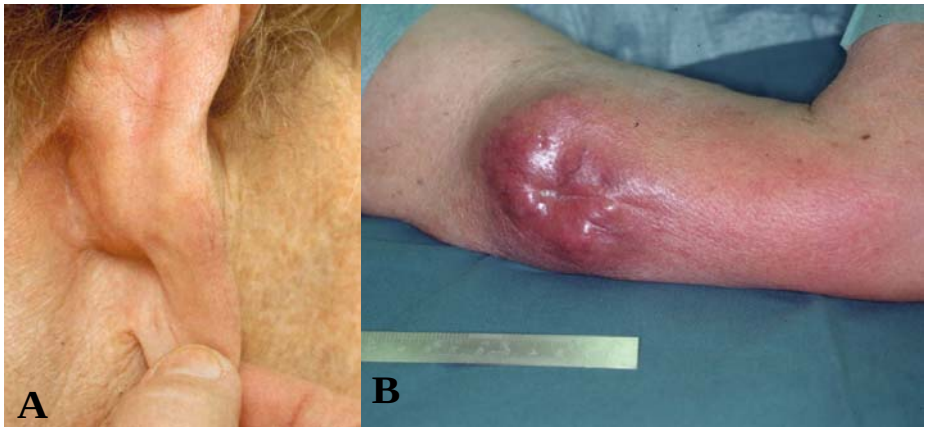
melanoma. In most published series MCC is not classified according to the TNM system. Yiengpruksawan has, however, proposed a staging system that has been widely recognised in the treatment of MCC (Yiengpruksawan et al. 1991). Briefly, stage I is defined by the absence of lymphadenopathy, stage II is defined by positive regional lymphadenopathy and stage III denotes evidence of distant metastases. This staging is presented in Table 3.

Table 3 Yiengpruksawan proposed a staging system with treatment recommendation

SLNB sentinel lymph node biopsy

	Stage	**Treatment recommendation**
I	Localised disease	Surgery: local excision > 2 cm margins, SLNB, radiation to the primary and the nodes
IA	< 2 cm primary tumour	
IB	≥ 2 cm primary tumour	
II	Regional lymph node involvement	Surgery: local excision > 2 cm margins, lymph node dissection, radiation therapy: adjuvant primary site and lymph node region
III	Distant metastases	Radiation and chemotherapy : palliative use

Figure 2 The clinical presentation of primary MCC. The tumour presents clinically in many ways. Picture A presents a small intradermal nodule in the back of the earlobe. Picture B presents a rapidly grown, in just three months, large MCC in brachium of a 78-year-old man. The scar in the middle of the tumour marks the primary intralesional excision.



2.6.2 Insidence and clinical behaviour of MCC

The reported annual incidence of MCC ranges from 0.2 to 0.45 per 100 000 (Chuang et al. 1990; Pan et al. 2002). It is 100 times as rare as melanoma (Landis et al. 1998). MCC is principally a disease of the Caucasian race. The annual age-adjusted incidence of MCC is 0.23 per 100,000 for whites and 0.01 for blacks (Miller and Rabkin 1999). The literature contains only single case reports of black Africans or African-Americans with MCC (Chao et al. 1990; Anderson et al. 1992; Matichard et al. 2002). Both sexes are affected, though earlier studies showed a slight male predominance (Feun et al. 1988; Boyle et al. 1995; Meeuwissen et al. 1995). The number of reported patient series is, however growing, and recent investigations have shown an equal distribution of the sexes or even a minor female prevalence (Ott et al. 1999; Akhtar et al. 2000; Colombo et al. 2000). MCC mainly affects the elderly, the mean age at presentation being about 75 years (Pergolizzi et al. 1997; Savage et al. 1997). Only a few cases occur before the age of 50, and is usually related to immunosuppression (Penn and First 1999).

MCC usually occurs in sun-damaged skin. The tumours are often found in close proximity to other lesions of actinically damaged skin, for instance, in cases of Bowen disease, squamous cell carcinoma, basal cell carcinoma, solar keratosis and lentigo maligna. The most common site is the head and neck region (Tai et al. 2000b). Pathogenetic factors such as UV irradiation may contribute to tumour development (Lawenda et al. 2001; Popp et al. 2002). Miller and Rabkin have calculated that the incidence of MCC and melanoma rises along with an increase in potential exposure to solar UVB. They found statistical significance between the incidence of MCC and UVB exposure ($p = 0.006$) the logarithms of the incidence rate of MCC increasing by 0.0021 for each unit of increase in the solar UVB index (Miller and Rabkin 1999).

The typical clinical course of the disease is rapid progression of the primary tumour with early and frequent metastasis to the regional lymph nodes. The sentinel lymph node biopsy (SLNB) technique has provided information on the metastatic dissemination at the presentation. In a meta-analysis of 60 MCC patients, Mehrany and co-workers detected 33% of the patients having metastatic dissemination in the sentinel lymph node at the presentation (Mehrany et al. 2002). Then again, Hohaus and co-workers in their 17 patients retrospective analysis reported only 3 (18%) of the patients at stage II at the presentation (Hohaus et al. 2003). Allen and co-workers detected 5/26 (19%) of the patients having positive lymph nodes at the presentation (Allen et al. 2001).

2.6.3 Co-existing malignancies

MCC has been associated with other skin tumours (squamous cell carcinoma, basal cell carcinoma) and haematological (B-cell) malignancies (Ziprin et al. 2000; Sinclair et al. 2003).

In addition, adenocarcinomas of the breast and ovaries have been shown to have an association with MCC. Co-malignancies, whether they appear before, after or simultaneously with MCC, are associated with higher MCC-specific mortality (Kurul et al. 2000; Brenner et al. 2001).

2.6.4 Immunosuppression: therapeutic and acquired

The incidence of MCC is abnormally high (8%) among immunosuppressed patients. Such patients are moreover younger, 49% being under the age of 50 (Gooptu et al. 1997; Buell et al. 2002). Weakened immunity increases the risk of MCC; HIV patients, for example, have a 13.4 times increased risk of acquiring MCC (Engels et al. 2002).

2.6.5 Prognosis

The prognosis is rather poor. The 2-year survival rate is 30 – 50% (Kokoska et al. 1997; Linjawi et al. 2001), Kokoska included 35 and Linjawi 10 patients in his study. Agelli and Clegg have studied the epidemiology of MCC in the United States in a patient population of 1034 by using Surveillance, Epidemiology, and End Results Program (SEER http://seer.cancer.gov). The 5-year survival rate in their study was 75%, 59% and 25% for localised, regional and distant MCC, respectively (Agelli and Clegg 2003). Overall survival (OS) is associated with the stage of disease at presentation and, furthermore, with sex but not with age. Female sex, localised disease and younger age were positive predictors of survival. (Hitchcock et al. 1988; Medina-Franco et al. 2001; Agelli and Clegg 2003). The risk of recurrence or metastasis was 19 times as great in sentinel lymph node biopsy positive patients as in biopsy negative patients ($p = 0.005$) (Mehrany et al. 2002).

Local recurrences are frequent, occurring in up to 44% of patients (Boyle et al. 1995; Haag et al. 1995; Pergolizzi et al. 1997). There have been some reports of lymph node metastasis alone, with no detectable primary tumour (Eusebi et al. 1992; Ferrara et al. 1997). Depending on the length of the follow-up period up to 36% of the patients develop regional lymph node metastasis (Gillenwater et al. 2001). Distant dissemination is not infrequent, up to 40-50% of patients developing visceral metastasis (Sibley et al. 1985; Raaf et al. 1986; Hanke et al. 1989), particularly to the lungs, liver and bone (Wynne and Kearsley 1988; Shack et al. 1994).

2.7 Surgical treatment

2.7.1 Current surgical options

Because of the rarity of the tumour, there is multitude of treatment protocols. However, the surgical treatment seems to be the corner stone of the different treatment protocols. Early, radical surgery is the recommended procedure for the treatment of primary MCC (Colombo et al. 2000;

Brissett et al. 2002). Margins of 2 – 5 cm are recommended for better local control. Nevertheless, consensus on the width of margins has not yet been reached. Yiengpruksawan and co-workers, O´Connor and Brodland reported better local control with margins of > 3 cm (Yiengpruksawan et al. 1991; O'Connor and Brodland 1996). Then again, there have been reports that larger free margins confer no advantage on survival (Gillenwater et al. 2001). Gillenwater and co-workers had sixty-six head and neck MCC cases included in their study, but only eighteen patient's data was sufficient for the statistical analysis. They state themselves that the small patient population in their study might explain this result.

Because lymph node metastases develop in approximately 50% of patients in the course of the disease, some authors recommend prophylactic lymphadenectomy in all patients (Kokoska et al. 1997; Lawenda et al. 2001). Then again, the patient materials are small: Lawenda had nine patients and Kokoska included 35 patients in the study. Silva and co-workers recommend lymphadenectomy for tumours with 10 or more mitoses per high-power field, in cases of lymphatic invasion, or when tumours are composed of small cells, that is, the small-cell subtype (Silva et al. 1984a).

Tumours in the midline present the problem of bilateral drainage, especially in the head and neck region. Goepfert and co-workers recommend that patients with such lesions undergo a bilateral neck dissection (Goepfert et al. 1984). Tumours arising in the head and neck region with parotid gland metastasis seem to have even poorer prognosis (de Mortillet et al. 1995). Elective lymph node dissection has been recommended for younger patients with large tumours or tumours of the head and neck region (Shaw and Rumball 1991; Victor et al. 1996; Allen et al. 1999).
It is nowadays accepted that patients with regional node metastases or local or regional recurrence should undergo excision of the primary lesion and lymph node dissection (Brissett et al. 2002). Adjuvant radiation therapy to the primary site and regional nodes is generally recommended in addition to lymph node dissection.
However, elective lymph node dissection increases mortality especially in an elderly patient population such as MCC patients.

2.7.2 Sentinel lymph node biopsy

The concept of sentinel lymph node was first presented by Cabanas in 1977 for penile carcinoma (Cabanas 1977). The theory of the procedure is based on the notion that lymphatic drainage from every anatomical region is regular. Hence, the trail and its end point, the sentinel node, can be illustrated by lymphoscintigraphy. The sentinel node reflects the state of the remaining lymph nodes in the lymph node basin. Removal of the sentinel node is called sentinel lymph node biopsy (SLNB). This technique is currently established treatment in melanoma and

breast cancer for staging purposes and for assessing the need for other treatments (Bilchik et al. 1998). According to the literature on MCC, the SLNB technique was used in approximately 130 cases between 1976 and 2004. Most reports advocate SLNB because morbidity is low, and because it provides an easy and reliable way of locating occult metastasis (Ames et al. 1998; Rodrigues et al. 2001; Mehrany et al. 2002). Recent studies have investigated the efficacy of SLNB in helping to determine whether lymph basin evacuation is necessary. These studies have been conducted on only a small series of patients, but even so, the results suggest that a negative sentinel node may obviate the need for neck dissection. SLNB is strongly advocated in the treatment and staging of individual tumours. Its use will improve the accrual of patients to adjuvant and further surgical treatment protocols (Wasserberg et al. 2000; Mehrany et al. 2002).

2.7.3 Mohs micrographic surgery

Mohs micrographic surgery is a surgical technique developed by Frederick Mohs in the 1930s at the university of Wisconsin. Nowadays it is used in few centers, however it is not a widely used technique.The original name, chemosurgery, was derived from the chemical paste containing 20% zinc chloride, which was applied to cancerous tissue to fix it in situ (Cottel et al. 1988). Briefly, the technique consists of debulking the tumour with a semisharp curette, in order to outline the skin tumour. Subsequently, a scalpel is used to remove the tissue in a horizontal fashion with 2 mm clinical margins. The tissue sample is taken to the laboratory for processing. The patient is bandaged and waiting for the microscopic results. The horizontal frozen sections are cut from the undersurface of the tissue parallel to the skin surface, rather than vertically. This method allows for microscopic examination of the entire deep and peripheral margins of the surgical specimen. The surgeon does the microscopic examination. These steps are then repeated until the margins are free from the tumour, and the defect is closed. Mohs micrographic surgery has been recommended as an advanced technique for local control, especially in areas calling for excellent cosmetic results (i.e. head and neck region) without compromising the principles of cancer surgery (O'Connor et al. 1997; Boyer et al. 2002). Then again, Brissett and co-workers reported inferior 2-year survival with patients undergoing Mohs micrographic surgery compared to patients who had wide local excision alone or wide local excision and lymphnode basin evacuation, 33%, 68% and 100% respectively (Brissett et al. 2002).

2.8 Oncological treatment

Chemotherapy is generally reserved for stage III (distant metastasis) cases of MCC. The carcinoma has often been shown to respond to chemotherapy but, as in SCLC, remission is brief. Recently, Waldman and co-workers reported a complete remission that lasted for 6 months. The remission was achieved through the use of high-dose polychemotherapy following autologous blood stem cell transplantation (Waldmann et al. 2000). Some months previously, Voog et al.

reported similar results, the rates of response to second-line (n = 33) and third-line (n = 10) chemotherapy being 45% and 20%, respectively (Voog et al. 1999). None of the chemotherapeutic protocol has, however, been able to achieve a significant increase in survival rate.

No standard chemotherapy protocol has yet been established for the treatment of MCC. Because of the morphological and immunohistochemical similarity of MCC to SCLC, chemotherapy has been performed with protocols based largely on agents active in SCLC. A wide variety of chemotherapeutic agents, including the cytostatic drugs cyclophosphamide, doxorubicin, epirubicin, vincristine, etoposide, cisplatine, carboplastin, 5-fluorouracil, dacarbazine, mitoxantrone, bleomycine and iphosphamide, have been discussed (Feun et al. 1988; Fenig et al. 1993; Voog et al. 1999; Tai et al. 2000b; Samonis et al. 2001). Unfortunately, reports to date consist of only small studies and anecdotal evidence. A few reports have shown markedly high mortality among MCC patients receiving chemotherapy for metastatic disease. Tai and co-workers reported seven (3.4%) toxic deaths among 204 cases (Tai et al. 2000b). Voog and co-workers gave even higher mortality, the rate of toxic death during first-line treatment being 7.7% in 101 patients (Voog et al. 1999).

MCC is a highly radiosensitive tumour. This has been demonstated by in vitro studies (Leonard et al. 1995). Many authors have recommended post-operative radiotherapy based on the retrospective comparison of patients treated with surgery alone with patients treated with surgery and post-operative radiotherapy (Bourne and O'Rourke 1988; Herbst et al. 2002). Radiation is most commonly used as an adjuvant therapy after surgery but it may also be used as the only treatment (Mortier et al. 2003; Pacella et al. 1988).

3. Tumorigenesis

3.1 General remarks

The origin of cancer can be traced to a single mutated cell. This one cell may give rise to a whole population of cells carrying the same mutation. If the mutation occurs in critical growth regulatory genes causing variations in cellular proliferation and survival, the process can lead to distorted cell physiology and thus to cancer. The alterations in these regulatory subjects can be caused by either intrinsic or extrinsic factors. In its essence, cancer is evolution at the microscopic level (Tsao 2001). Narrowed down to the molecular level, cancer arises due to errors in the regulation of the cell cycle. Hanahan and Weinberg have defined six changes in cell physiology that can be identified in cancer formation (Hanahan and Weinberg 2000): self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, avoidance of programmed cell death, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis. Immunohistochemical detection of these changes within a tumour sample is a merely due to in the cell cycle regulation.
Cancer thus originates from gene or chromosome changes. The aberrations may vary from single mutations to chromosomal distortions. In some tumours specific changes e.g. translocations of certain chromosomes have been identified, and they can be employed in differential diagnostics, like in Ewing sarcomas (Tarkkanen and Knuutila 2002). In many tumours no specific changes have, however yet been identified, as in the case of MCC. There are a wide variety of techniques to study gene and chromosomal changes, like comparative genomic hybridisation (CGH) and loss of heterozygosity (LOH).

3.2 Growth regulation

Tumour growth reflects the balance between cellular proliferation and cell loss (apoptosis). The cell cycle, also called the proliferative cycle or mitotic cycle, has four phases: Gap 1 (G1) (presynthetic), S (DNA synthesis), Gap 2 (G2) (premitotic), and M (mitotic). Quiescent cells are in a resting state called G0. During the S phase, the genome is duplicated in the normal cell cycle. In the mitotic (M) phase, the duplicated genome is separated into two equal cells containing an identical genome to the parent cell (Hall and Levison 1990). The integrity of the genome is controlled in many ways, mainly at the restriction points, or cell cycle checkpoints. One such checkpoint occurs just before the cell enters the S phase and another before the cell enters the M phase. The cell cycle is highly regulated by chemical factors that can either stimulate or restrain cell proliferation. Normal cells are regulated by extracellular growth signals, mainly during the G1 phase. For a cell to develop naturally, positive and negative regulators of the cell cycle must proceed in a very carefully controlled manner. Cancer can arise from overexpression of positive regulators, e.g. cyclin-dependent kinases (CDK) and their cyclin partners, or from

underexpression of negative regulators, e.g., CDK inhibitors (CDKI) (Sherr and Roberts 1995; Sherr 1996). Due to disturbances in normal apoptotic pathways, genetic changes do not result in cell cycle arrest, but the altered cell with the altered genome continues the cell cycle.

3.2.1 Cyclin-dependent kinases and cyclins

Several kinase complexes associating with cyclins and their catalytic subunits, e.g., cyclin-A, regulate cellular proliferation. Cyclin-dependent kinases (CDKs) are positive regulators or accelerators of the cell cycle. Cyclins are divided by their function and by their appearance in the cell cycle into G1 and mitotic cyclins. Cyclin-A and cyclin-B are considered as mitotic cyclins. During the cell cycle, they are expressed in oscillatory fashion, accumulating progressively through the interphase and disappearing at the end of mitosis (Swenson et al. 1986; Pines and Hunter 1989; Clarke et al. 1992). Cyclins function together with a protein kinase partner, CDK, forming an active complex. In higher eukaryotes, cell cycle progression is controlled by the CDKs, whose activity is regulated in part by their association with distinct cyclins at different times in the cell cycle. Deregulation of cell cycle regulators may be connected with tumorigenesis. Overexpression of cyclins can cause uncontrolled growth of cells, resulting in an unfavourable course of disease (Murakami et al. 1999; Reissman et al. 1999; Liang et al. 2000).

3.2.2 Cyclin-A

Progression through the cell cycle is driven by the sequential and periodical activation of cyclin/CDK complexes. Entry into and progression through the S phase is promoted by activation of cyclin-A/CDK2 and cyclin-E/CDK2. Overexpression of cyclin-A is thus a reflection of errors in cell cycle control. Cyclin-A activates two different cyclin-dependent kinases, CDK1 and CDK2. In the S phase, its function, together with CDK2 (cyclin-A/CDK2 complex), is to induce phosphorylation of the components of DNA replication (Jeffrey et al. 1995). Mitosis is controlled by the specific and timely degradation of key regulatory proteins, notably the mitotic cyclins (cyclin-A and cyclin-B) that bind and activate the CDKs. The function of cyclin-A in mitosis is to stabilise cyclin-B, controlling its half-life and preventing the degradation of cyclins. In animal cells, cyclin-A is always degraded before cyclin-B. Cyclin-A is first expressed at the G1/S boundary and is gone before metaphase. It can activate both CDK1 and CDK2, and function in both S-phase and mitosis (Marcote et al. 1992; Pagano et al. 1992). Removal of cyclin-A is carried out by ubiquitin-mediated proteolysis (Glotzer et al. 1991).

Cyclin-A has proved to be of prognostic value in different types of carcinomas and sarcomas (Furihata et al. 1996; Volm et al. 1997; Noguchi et al. 2000; Kyushima et al. 2002). Over-expres-

sion of cyclin-A was an independent marker for a poorer prognosis in early stage breast carcinoma (Michalides et al. 2002). High labelling index of cyclin-A expression correlated with overall survival in squamous cell carcinoma of the mouth (Chen et al. 2003). In soft tissue sarcomas a correlation was established between high proliferation rate measured by cyclin-A expression and a favourable response to chemotherapy (Huuhtanen et al. 1999).

3.4 Invasion and metastasis

A tumour's malignancy, morbidity and mortality are defined largely by its capacity to invade and metastasise. Death from cancer is usually due to metastasis. Neovascularisation is essential to the development of the primary tumour, as well as, for the metastatic process. The metastasis process includes the development of neovascularisation, through which the tumour cells extravasate in new locations and initiate growth and metastasis. Blood flow and other mechanical factors influence the delivery of cancer cells to specific organs, whereas molecular interactions between the cancer cells and the new organ influence the probability of the cells growing there (Chambers et al. 2002; MacDonald et al. 2002). Fortunately, not all the cells that escape from the primary tumour form metastasis.

Tumour cells interact with the extracellular matrix (ECM). The ECM surrounds the cells and, as well as holding the cells and organs together, it serves as a mediator of receptor-induced interaction between the cells. Adhesive interactions of cells with the ECM play an important role in the dynamic changes involved in morphogenetic processes such as neurite outgrowth, branching morphogenesis of the epithelium, and also angiogenesis (Bischoff 1995; Gumbiner 1996).

3.4.1 Cyclooxygenase-2 (Cox-2)

Cyclooxygenase (Cox) is the key enzyme in the conversion of arachidonic acid to prostanoids (Vane et al. 1998)**.** Two Cox genes exist, Cox-1 and Cox-2. Cox-1, a constitutive enzyme produced continuously in most tissue types, is probably responsible for the production of prostanoids under physiological conditions. Cox-2 is undetectable in most normal tissues but can be induced in various cell types by inflammation and in carcinogenesis (Taketo 1998b; Taketo 1998a). Several types of carcinomas have been shown to express elevated levels of Cox-2 mRNA and protein (Koki et al. 2002)**.** Cox-2 overexpression by malignant cells has been shown to enhance cellular invasion, induce angiogenesis, regulate anti-apoptotic cellular defenses and promote immunological resistance through the production of prostaglandin E2 (Tsujii et al. 1997; Leahy et al. 2000; Koki et al. 2002). Most Cox-2 studies have focused on colorectal cancers. Fosslien showed that Cox-2, but not Cox-1, is highly expressed in human colon carcinoma, squamous cell carcinoma of the oesophagus, and skin cancers (Fosslien 2000). In human colorectal carcinomas, Fujita and co-workers found that Cox-2 levels were significantly higher

in large tumours and in tumours with deeper invasions but that the level did not correlate with the metastasis rate. Further they suggest that larger carcinomas produce more Cox-2 to support their own growth and that Cox-2 inhibitors may be effective agents of carcinoma growth suppression (Fujita et al. 1998).

A growing number of reports describe the therapeutic effects of Cox inhibitors in chemoprevention as well as tumour regression in experimental animal models. Fischer has studied the role of arachidonic acid and its products in chemically and UV-light-induced skin carcinogenesis in murines, with the emphasis on determining the importance of prostaglandins (Fischer 2002). In her study, dietary administration of the selective Cox-2 inhibitor celecoxib prevented the development of UV-induced skin cancers by up to 85% in murines. In addition, celecoxib caused regression of pre-existing tumours. Robertson and co-workers reported that a 35-day course of ibuprofen in rats with induced mammary carcinomas resulted in significant reduction of tumour volume ($p < 005$) (Robertson et al. 1998). In the literature, there are no reports on Cox-2 expression in MCC or the role of cyclooxygenase inhibitors in the treatment of MCC.

3.4.2 Tenascin-C

Tenascin-C (Tn-C), a modular hexameric ECM glycoprotein, is the founding member of the tenascin family of ECM proteins. Tenascin is synthesised by fibroblasts and is thought to play a role in cell adhesion and motility, supporting cell growth, tissue modelling and the formation of demarcation lines along the tissue lines (Inaguma et al. 1988). Tn-C was first observed in 1983 and named glioma mesenchymal ECM antigen (Bourdon et al. 1983). A large glycoprotein of the ECM, it is expressed transiently during organogenesis and in adult tissues in regions of inflammation, in wound healing and in neoplasia (Natali et al. 1991; Latijnhouwers et al. 1998). It is absent from or reduced in fully developed organs (Chiquet-Ehrismann and Chiquet 2003).

Tn-C is composed of six identical subunits made up of repeated sequence motifs that fold independently into small globular domains. The most prominent structural domains are tenascin-type epidermal growth factor (EGF)-like repeats, fibronectin type III repeats, and the fibrinogen globe. A number of variants of Tn-C generated by alternative mRNA splicing of the fibronectin type III repeats have been described (Chiquet-Ehrismann 1995; Chiquet-Ehrismann and Chiquet 2003). As the molecule is composed of EGF–like repetitions that can bind to EGF receptors of tumour cells, it is thought that tenascin may contribute to invasion and metastasis (Jones et al. 1988).

Tumorigenesis involves many processes revealed by Tn-C expression. These include angiogenesis, proliferation, and inflammatory processes (Chiquet-Ehrismann and Chiquet 2003).The

intensity of Tn-C expression correlates with poorer prognosis in tumours of different origin, e.g., breast, skin and brain (Jahkola et al. 1996; Tuominen et al. 1997; Herold-Mende et al. 2002). Tn-C tends to accumulate in the invasion border, a staining pattern that correlates with poorer prognosis (Jahkola et al. 1998). In some reports, Tn-C negativity forecasted a poorer prognosis (Sugawara et al. 1991). This may be explained by the finding that Tn-C is absent from or at least reduced in such tumours that have reached their full metastasis and invasive potential.

In skin tumours, enhanced expression of Tn-C was noted along with a growing degree of malignancy. Expression was moderate in benign naevi and strongest in invasive malignant melanomas (Tuominen and Kallioinen 1994). Basal cell carcinomas showed marked staining as intense, well-defined bands around neoplastic islands (Stamp 1989; Saitoh et al. 1996). In the literature, there are no reports on Tn-C expression in MCC.

AIMS OF THE STUDY

The main purpose of this study was to identify tumour-related and molecular markers for predicting the behaviour of MCC. Because of the rarity of this tumour and because most of the published series are small, guidelines for and consensus on treatment are lacking. A relatively large number of MCC specimens with clinical data were therefore collected. The specimens were examined by immunohistochemistry and comparative genomic hybridisation and the results were compared with the clinical outcome of individual patients.

The study had five specific aims:

1. to define tumour-, patient- and treatment-related prognostic factors for survival (Study I)

2. to examine the DNA copy number changes in MCC with a view to assessing their correlation with the course of disease (Study II)

3. to investigate the expression of cell cycle enhancer, cyclin-A, and its prognostic significance (Study III)

4. to shed light on the role of Cox-2 expression and its relevance to therapeutic Cox-2 inhibition (Study IV)

5. to explore the expression of tenascin-C and evaluate its role as a prognostic marker (Study V)

MATERIALS AND METHODS

1. Clinical material (Studies I-V)

The study was approved by the Ethics Committee of the Department of Surgery, Helsinki University Central Hospital (no. 296/E6/2001). Tissue specimens were obtained at operations performed at the Department of Plastic Surgery, Helsinki University Central Hospital, and at the Department of Surgery, Vaasa Central Hospital, in 1987-2003. The original patient material consisted of 34 patients. During the study period, four more patients were diagnosed with MCC and they were included in this study. Altogether 38 patients samples and clinical data were evaluated. Table 4 presents the clinicopathologic characteristics of the included patients. If the patient had been referred to either of the above hospitals for a larger operation, the primary care centre was contacted in order to obtain the primary sample for analysis. The clinical appearance of the primary tumour was recorded as described in the documents completed by the referring doctor.

The tissue samples were routinely embedded in paraffin. They were then processed routinely at the Department of Pathology, Helsinki University Central Hospital, stained with HE, CK-20 and TTF-1 to confirm the diagnosis, for light microscopic study. The TTF-1 was negative in all samples. All stained samples were evaluated by two pathologists. Tumour size, that is, the greatest surface dimension, was measured from HE-stained slides and documented as < 2 cm or ≥ 2 cm. Invasion to the underlying tissue was recorded; if not present, the tumour was regarded as a superficial carcinoma.

The clinical data were gathered from the surgical, oncological and pathological databases of both hospitals. The following patient and treatment characteristics were recorded: age, sex, primary tumour location, primary tumour size, invasion to subcutaneous tissue, date of initial surgery, method of initial surgery (simple excision, excision and split thickness skin graft or reconstruction with local flap), disease status at presentation (regional metastasis, distant dissemination), co-existing diseases, previous malignancies, reason for re-operation (positive resection margins, local recurrence, metastatic course of disease), date of re-operation, method of re-operation, post-operative radiation therapy, adjuvant chemotherapy, and date of death.

Table 4 The clinicopathological characteristics of 38 MCC patients

No. of patients	38
Sex (%)	
Female	23 (61%)
Male	15 (39%)
Age (years)	
Mean	80
Range	59 – 100
Tumour location	
Head and neck	18 (47%)
Extremities	13 (34%)
Trunk	3 (8%)
Unknown primary	4 (10%)
Tumour size	
Median	2.5 cm
Range	3 mm – 6.5 cm
< 2 cm	13
≥ 2 cm	21
Recurrence	11 (29%)
Metastasis	17 (45%)
Follow-up	
Median	4 years
Range	8 d – 14.3 yr
Died during follow-up	18 (47%)

2. Comparative genomic hybridisation (Study II)

DNA was extracted from paraffin-embedded tissue sections following the procedure reported by Isola and co-workers (Isola et al. 1994). Comparative genomic hybridisation (CGH) was performed using direct fluorochrome-conjugated DNA on all samples according to the protocol described elsewhere, with minor modifications (Larramendy et al. 1997). Briefly, tumour DNA and reference DNA (genomic DNA from peripheral blood leukocytes from normal donors) were labelled by nick translation with fluorescein-iso-thiocyanate (FITC)-conjugated dCTP and dUTP (DuPont, Boston, MA, USA), and TexasRed-conjugated dCTP and dUTP (Dupont), respectively, to obtain fragments ranging from 600 to 2000 bp as previously reported (Larramendy et al. 1998b). The hybridisation mixture consisted of 400 ng of tumour DNA, 400 ng of reference DNA, and 10 ìg/ of unlabelled human Cot-1 DNA (Gibco/BRL, Life Technologies, Gaithersburg, MD, USA) dissolved in 10 ìl of hybridisation buffer (50% formamide, 10% dextran sulphate, 2 x SSC). The hybridisation mixture was denatured at 75°C for 5 minutes and hybridised to a slide with normal metaphase spreads denatured in 70% formamide/2 x SSC (pH 7) at 68°C for 2 minutes. Hybridisation was carried out at 37°C for 48 hours. The slides were then washed three times in 50% formamide/2 x SSC (pH 7), twice in 2 x SSC, and once in 0.1 x SSC at 45 °C, followed by 2 x SSC, 0.1M NaH_2PO_4-0.1M Na_2HPO_4-0.1% Nonidet P-40 (pH 8) and distilled water at room temperature for 10 minutes each. After air-drying, the slides were counterstained with 4', 6-diamidino-2-phenyl-indole-dihydrochloride (DAPI) (Sigma Chemical Co., St. Louis, MO, USA), and mounted with an antifading medium (Vectashield®, Vector Laboratories, Burlingame, CA, USA).

2.1 Digital image analysis

The extent of hybridisation was analysed with an Olympus fluorescence microscope and the ISIS digital image analysis system (MetaSystems GmbH, Altlussheim, Germany), based on an integrated high-sensitivity monochrome charge-coupled device camera and automated CGH analysis software. Three-colour images (green for tumour DNA, red for reference DNA, and blue for counterstaining) were acquired from 12 metaphases per sample. The chromosomal regions were interpreted as over-represented when the green-to-red ratio exceeded 1.17 (gains) or 1.5 (high-level amplifications) and as under-represented (losses) when the ratio was less than 0.85. In each CGH experiment, a negative (peripheral blood DNA from normal donor) control and a positive (tumour DNA with known copy number changes) control were included and run simultaneously with the tumour samples. Telomeric and heterochromatinic regions were excluded from the analysis when they appeared as the sole aberration present in the sample, as these regions cannot be evaluated reliably by CGH (Kallioniemi et al. 1994a; Larramendy et al. 1998a). DNA copy number changes involving 16p and 19 were excluded from analysis unless they represented high-level amplification (Kallioniemi et al. 1994b; Kallioniemi et al. 1995). All

results were confirmed using a 99% confidence interval with 1% error probability. Briefly, intraexperimental standard deviations for all positions in the CGH ratio profiles were calculated from the variation in the ratio values of all homologous chromosomes in the experiment. Combining them with empirical interexperimental standard deviation and estimating the error probability based in the t-distribution then computed the confidence intervals for the ratio profiles.

3. Immunohistochemistry (Studies III-V)

Four-micron sections were cut from paraffin-embedded blocks, deparaffinised in xylene and rehydrated in a series of graded alcohols. The sections were pre-treated in a microwave oven in 10 mmol/L citrate buffer, pH 6.0, at 600 watts for 20 minutes. Endogenous peroxidase activity was blocked in 0.5% H_2O_2 for 30 minutes. Primary antibodies were applied overnight according to the following list:

- cyclin-A	Novo Castra Laboratories, Newcastle upon Tyne, UK	1:100 (Study III)
- Cox-2	160112, Cayman Chemical Co., Ann Arbor, MI, USA	1:200 (Study IV)
- Tn-C	clone DB7, Biohit Diagnostics Oy, Helsinki, Finland	1.2000 (Study V)

Binding of the primary antibody was detected with the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA) according to the manufacturer's instructions, and the sections were slightly counterstained with haematoxylin. All antibodies were monoclonal. The positive controls used throughout the staining procedure were:

- cyclin-A	gut mucosa
- Cox-2	colon carcinoma
- Tn-C	breast carcinoma

Omisson of primary antibodies was used as a negative controls. Positivity and the percentage of positively stained cells were determined from one slide per tumour. Five representative high-power fields were chosen, and the distribution of immunoreactivity was established by quantifying the percentage of positive nuclei, which was expressed as the labelling index (Tang et al.) only for cyclin-A (Study III); for cyclin-A, the cut-off point for strong and weak expression for statistical analysis was 20%.
For Cox-2 and Tn-C (Studies IV and V), a cytoplasmic staining pattern was evaluated. For Cox-2, positivity was graded as very low, low or moderate. For Tn-C, scores were low, moderate or

strong intensity. Whether the different degrees of positivity correlated with the outcome was studied by quantifying the staining intensity.

4. Statistical analysis (Studies I-V)

Statistical analyses were conducted with NCSS 2000 (NCSS Statistical Software, Kaysville, UT, USA) software except in Study IV (Cox-2), in which the calculations were made with StatView 5.0® (Abacus Concepts Inc., Berkeleu, California, USA). The selected level of significance was $p < 0.05$.

Study I: Multivariate analysis was performed by the Cox proportional hazard ratio method for age, sex, primary tumour size (< 2 cm and ≥ 2 cm), method of initial surgery (simple excision, excision and split thickness skin graft or reconstruction with local flap), development of metastatic disease (yes/no), development of local recurrence (yes/no). Overall survival (OS) was calculated by the Kaplan-Meier method. OS was calculated by the same method according to primary tumour size. For OS, the time interval was the time from initial surgery to the time of death of any cause or to the date of the last follow-up (30th November 2001). The odds ratio was calculated for primary tumour size (< 2 cm or > 2 cm) and development of metastasis (yes/no).

Study II: The association between tumours with no DNA changes, tumours with changes in chromosomes 6, 1, 5, 13 and 4 and with clinical outcome was tested by Fisher's exact test. The end-point for surveillance was chosen to detect recurrences or metastasis, or both.

Study III: The correlation between cyclin-A and local recurrence, metastatic dissemination and invasion to the subcutaneous tissue and OS was calculated by the Mann-Whitney U test. The correlation between tumour size and cyclin-A was determined by the Spearman rank order correlation test (cut-off point 2 cm). Univariate analyses were conducted by Fisher's exact test, which was used to compare moderate and overexpression (cut-off point 20%) of cyclin-A and the above variables.

Study IV: Clinical data were analysed against Cox-2 staining by Student's t-test and Fisher's exact test. Cox-Hazard analysis served for survival analysis.

Study V: The Chi-Square test was used to analyse clinical data against Tn-C staining.

RESULTS

At HE staining the MCC samples studied here showed dermal involvement with repeated extension to subcutaneous tissue. The tumours consisted of small blue cells with sparse cytoplasm; nuclei were medium in size and mitoses were abundant. The result of immunohistochemical analysis for CK-20 was positive in all samples, whilst that of TTF-1 was negative in all samples.

Table 5 presents the patient´s treatments. Table 6 presents an overview of the clinicopathological descriptions of individual studies. Figure 3 presents examples of immunohistochemical stainings in the studies III- V.

1. Clinicopathologic data (Study I)

1.1 Survival

The median OS rate was 2.7 years (range, 2.1 to 10.5 years). The median OS rate for tumour size ≥ 2 cm was 1.7 years (range, 43 days to 2 years) and for tumour size < 2 cm 4.48 years (range, 2.1 years to 14.3 years). The 2-year survival rate for tumour size < 2 cm was 70% and 5-year survival was 55%; in the group tumour size ≥ 2 cm, the 2-year survival rate was zero.

1.2 Prognostic factors for survival

Poor prognostic factors for OS were: male sex ($p < 0.05$), large size of the primary tumour (≥ 2 cm) ($p < 0.009$) and metastatic dissemination at any time point during follow-up ($p < 0.009$). The only favourable prognostic factor was reconstruction with a split thickness skin graft or a local flap ($p < 0.02$). The odds ratio for tumour size ≥ 2 cm and developing metastasis was 2.08.

Table 5 The treatment of 38 MCC patients, some of the patients had several treatments

Surgical treatment	38
Simple excision and direct closure	19
Excision and reconstruction with split thickness skin graft/local flap	15
Lymphnode biopsy	4
Excision and reconstruction with microvascular flap	1
Simple excision and regional lymph node dissection	1
Regional lymph node dissection (unknown primary)	2
Oncological treatment	14
Radiation therapy	12
Adjuvant chemotherapy	1
Radiation therapy and adjuvant chemotherapy	1

Table 6 Overview of patients and tumour descriptions in individual studies

	Study I	Study II	Study III	Study IV	Study V
No of patients	34	19	25	22	25
Sex					
Female	22	9	13	13	15
Male	12	10	12	9	10
Age (years)					
Mean	76.4	68	76	76	77
Range	59 – 97	59 – 97	59 – 97	59 – 97	59 – 100
Tumour location					
Head and neck	16	8	12	11	11
Extremities	11	8	10	8	11
Trunk	3	3	3	3	3
Unknown primary	4	-	-	-	-
Tumour size (cm)					
Median	2	2.5	2.5	1.9	2.5
Range	0.3 - 6	0.8 – 6.5	0.4 – 6.5	0.4 – 6.0	0.8 – 6.5
< 2 cm	11	9	13	13	11
≥ 2 cm	19	10	12	9	14
Metastasis	14	9	11	8	11
Recurrence	10	8	8	8	9
Follow-up (years)					
Median	4	2.7	3	3.2	3.2
Range	7 d – 14.3	7 d – 10.5	7 d -10.5	7 d - 11	7 d – 11

2. Comparative genomic hybridisation (Study II)

Thirteen (68%)of the nineteen samples showed changes, with a mean value of 5.5 ± 1.1 aberrations per sample (range, 1-12); six (32%) did not show any aberrations. The frequency of DNA copy number gains was twice that of losses (gains: losses=1.8:1). Table 7 presents the DNA sequence copy number changes as well as the clinical outcome of the patients.

2.1 Most frequent gains of DNA sequence copy number

Location	Number (%)
- chromosome 6	8 (42%)
- chromosome 1	7 (37%)
- chromosome 5	6 (32%)
- chromosome 12	4 (21%)

2.2 The most frequent minimal common regions of gains

Location	Number (%)
- 6pterqter	8 (42%)
- 1q11q31	6 (32%)
- 5p	6 (32%)
- 1q32qter	5 (26%)

2.3 Most frequent losses of DNA sequence copy number

Location	Number (%)
- chromosome 13	4 (21%)
- chromosome 4	3 (16%)

2.4 Most frequent minimal common regions of losses

Location	Number (%)
- 13q13q31	4 (21%)
- 4q	3 (16%)
- 16q	2 (11%)

2.5 Relation to tumour size and metastasis

In six of the tumours, no DNA copy number changes were detected. Only one (17%) of the tumours with no DNA copy number changes progressed to metastatic disease. There were nine small tumours (< 2 cm), five (55%) of them showed no DNA changes.
All eight samples with DNA copy number gains in chromosome 6 were seen in large tumours (≥

2 cm). Five (63%) of those patients with gains in their tumours in chromosome 6 developed a metastatic course of disease. Six samples showed gains in chromosome 1. Four (66%) of those patients with tumours with gains in chromosome 1, advanced to metastatic dissemination.
All the five samples with losses in chromosome 4 were large tumours (≥ 2 cm). There were four samples with losses in chromosome 13. Samples with losses in chromosomes 13 and 4, 50% and 66% progressed to metastatic dissemination, respectively.

Table 7 Overview of DNA copy number changes revealed by CGH, in 19 primary MCC samples with clinical outcome, R recurrence, M metastases, N no recurrence/metastases

Sample number	CGH results	
1	No changes	N
2	No changes	N
3	rev ish enh(1q,5p),dim(7q,13q13q32)	N
4	No changes	N
5	rev ish enh(1,5p,17,21),dim(5q,7q,10p,13),amp(4p)	M
6	dim(X)	R/M
7	No changes	R
8	rev ish enh(2q32qter,5pterq15,6),dim(4,16q),amp(6p)	M
9	rev ish enh(1,3q,5p,6,8),dim(2,3p,4,7p,11p,13pterq31)	N
10	rev ish enh(6,12,19,21)	R/M
11	rev ish enh(1pterp33,1q11q31,16p)dim(1p31p11,1q32qter,4q,16q),amp(1q22q24)	R/M
12	rev ish enh(1q,6,9,11,12,16p),dim(8p,10q)	R/M
13	rev ish enh(6)	M
14	rev ish enh(5p,6,12)	R
15	rev ish enh(5p,6),amp(5p)	N
16	rev ish enh(1,4p,6,7,9,12,14,19,20,22),dim(13q13q31,18q12-qter)	R/M
17	No changes	M
18	No changes	R
19	rev ish dim(8pterq13)	N

2.6. Statistical analysis

No significant statistical correlation was found between the CGH aberrations and clinical features ($p > 0.05$). However, the Relative Risk for developing metastatic dissemination in the presence of DNA copy number changes was 3.0.

3. Cyclin-A (Study III):

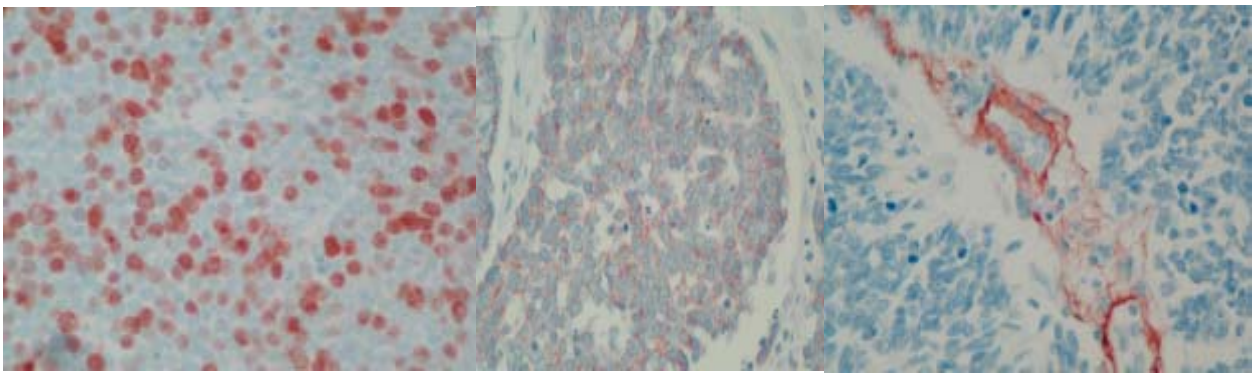
All samples showed positive staining for cyclin-A. Positivity ranged from 5.4% to 69.2%, the mean value of positive cells being 25% and the median 21%. Expression of cyclin-A was similar in the smallest and the largest tumours (19.6% and 19.7%, respectively). Overexpression

of cyclin-A was determined in 20% of the positive cells; hence, two comparable cohorts were formed.

3.1 Relation to tumour size and metastasis

There was no difference in expression of cyclin-A between the large (≥ 2 cm) and the small tumours (< 2 cm), 28% and 25%, respectively. Of the eleven large tumours, six (58%) showed overexpression of cyclin-A. Eleven (44%) patients developed metastatic course of disease. Six (54%) of the eleven tumours with metastatic dissemination showed staining positivity > 20%. In tumours with metastatic expansion the mean expression of cyclin-A was 30% and in the non-metastatic tumours 22%.

Figure 3 Immununohistochemical staining results of primary MCC for studies III - V. **A** 25 % of cyclin-A expression, original magnification 200x **B** moderate Cox-2 expression, original magnification 200x **C** strong Tn-C expression around the vascular sturctures within the tumour tissue,original magnification 200x.



A **B** **C**

3.2 Statistical analysis

Cyclin-A expression did not correlate with local recurrence ($p > 0.05$), metastatic dissemination ($p > 0.05$) or invasion of subcutaneous tissue ($p > 0.05$). Nor was the correlation between cyclin-A and tumour size significant ($p < 0.61$). There was no correlation between OS and cyclin-A expression ($p < 0.83$). The correlation coefficient was $r^2 = 0.11$.

4. Cox-2 (Study IV)

Staining for Cox-2 was positive in 15 (68%) of 22 samples. On overall the expression was low. Staining intensity was moderate in 14%, low in 18%, and very low in 36% of samples. In all samples, the staining pattern was scattered and did not cover the whole tumour. Eight (73%) of the 11 tumors located in the head and neck region expressed Cox-2, and six (75%) of the eight

tumors located in the extremities showed positive staining. Only one of the three tumours located in the trunk was positive for Cox-2; none of the three superficial carcinomas showed Cox-2 expression.

4.1 Relation to tumour size and metastasis

In the study material, there were 13 small (< 2 cm) and 9 large (≥ 2 cm) tumours. Nine (69%) of the small tumours and six (67%) of the large tumours were Cox-2 positive. Of interest is that all three tumours with moderate staining intensity were small; four (57%) of the seven Cox-2 negative cases were small tumours.
Eight of the patients developed recurrences and another eight patients developed metastasis. Five patients (63%) in both groups (recurrence and metastasis) showed positive Cox-2 staining, the metastasis rate in the Cox-2 negative cases was 42% .

4.2 Statistical analysis

Cox-2 staining, whether positive or negative, or the different degrees of positivity (moderate, low or very low), had no significant statistical correlation with clinical parameters (age, sex, tumour location, tumour size, invasion, recurrence or metastasis.

5. Tenascin-C (Study V)

Staining for Tn-C was positive in 17 (68%) and negative in eight (32%) of the 25 samples. Staining intensity was strong in nine, moderate in four and low in four samples. Staining was patchy and occurred mainly in the borders of the tumour, especially towards the subcutaneous fat. Except in the lining of vascular structure, Tn-C staining was virtually nonexistent within the tumour tissue. In nine tumour samples, the connective tissue septae were stained. Staining intensity around septae tended to be strong. In the tumour cells, there was no cytoplasmic staining. The staining was continuous, with a flame-like pattern and extensions to the surrounding tissues.

5.1 Relation to tumour size and metastasis

Of the eleven small tumours, six (55%) did not express Tn-C. The large tumours expressed positive staining in twelve (86%) of the fourteen tumours. Of the eleven tumours disseminating

to metastases, nine (82%) were Tn-C positive. Interestingly, six of the eight negative tumours were small. The very smallest ones were usually Tn-C negative.

5.2 Statistical analysis

There was a statistically significant association between the positivity of Tn-C expression and tumour size. A correlation with large tumour size (≥ 2 cm; $p = 0.032$) was established. Staining intensity (strong, moderate, low) did not correlate with clinical parameters, i.e. (age, sex, tumour location, invasion, recurrence or metastasis).

The immunohistochemical results of Studies III, IV and V are summarised in Table 8. Intensity (strong, moderate, low) did not correlate with clinical parameters, i.e. (age, sex, tumour location, invasion, recurrence or metastasis).

Table 8 Overview of immunohistochemical results in Studies III, IV and V and their relations to tumour size and metastasis.

	Cyclin-A	Cox-2	Tn-C
Tumour size < 2 cm			
mean expression	25%		
	< 20% expression 7 (54%)		
	≥ 20% expression 6 (46%)		
no. of positive samples		9 (69%)	5 (54%)
Tumour size ≥ 2 cm			
mean expression	28%		
	< 20% expression 5 (42%)		
	≥ 20% expression 7 (58%)		
no. of positive samples		6 (67%)	12 (86%)
Metastatic tumours			
mean expression	30%		
	< 20% expression 5 (45%)		
	≥ 20% expression 6 (55%)		
no. of positive samples		5 (63%)	9 (82%)
Non-metastatic tumours			
mean expression	22%		
	< 20% expression 7 (50%)		
	≥ 20% expression 7 (50%)		
no. of positive samples		10 (71%)	8 (57%)
Recurrence			
mean expression	-		
no. of positive samples		5 (63%)	6 (67%)

Discussion

The prognosis and treatment of MCC is based presently on the presence or absence of metastases. The aim throughout the study was to define easy and reliable markers to predict the course of disease. These markers should be significant, independent and clinically important. They should be of practical use, and their determination should be affordable in everyday practice. In the quest for such markers, standard immunohistochemistry was performed for primary MCC samples using antibodies that have proven their value in cancer research but that could also expand our understanding of the natural course of MCC in a wide span. To further elucidate the molecular events important for MCC carcinogenesis the tumours were also studied by CGH to reveal what chromosomal imbalances are common, and might therefore give clues to which genes are of importance. To be able to compare the possible use of such markers clinically well-documented and studied material is needed. In this study, the clinical data (study I) was compared against the CGH results (study II) and furthermore to the expression level of some known prognostic markers (studies III – V).

1. Prognosis based on clinical findings

The Finnish Cancer registry received 141 notifications of MCC in Finland in 1987–2001 (Dr. Risto Sankila, Finnish Cancer registry, oral communication). In a population of 5 000 000, this gives an annual prevalence of 0.2/100 000. In this study, 38 of these cases were included from two centres in Finland. The tumour presented clinically in many ways, at least in the early stages of the disease. In 26 cases, the referring doctor had initially described the tumour as a tumour or intradermal lump; in six of these, ulceration was present. In 11 cases, the tumour resembled an epidermal cyst and in one case, it was mistaken for basal cell carcinoma. Co-existing malignancies of the patients are given in Table 9.

The female predominance was strong in this study (22 women, 65%/12 men, 35%). One possible explanation for this unusually marked female predominance might be age, as women are known to outlive men, and patients presenting with MCC tend to be elderly.

1.1 Size and prognosis

MCC has a tendency to progress rapidly, and in just a few months, the tumour may attain a large diameter. Therefore, early diagnosis and surgery are strongly advocated. This study established a close correlation between OS and tumour size. The OS rate is largely influenced by tumour size

(≥ 2 cm, p < 0.009), a finding that is in line with previous studies (Tai et al. 2000a). In fact, the cut-off point 2 cm used in this work is identical to that used in the TNM classification,where T1 tumours are ≤ 2cm. In this study, the OS was 2.6 times higher in those patients who presented with small tumours (< 2 cm). Furthermore, in this study the odds ratio for larger tumours proceeding to metastatic course of disease was two-fold. Figure 4 presents overall survival according to the tumour size.

Table 9 Co-existing malignancies of the patients.

Co-existing malignancy	No. of patients
Prostate carcinoma	2
Chronic lymphatic leukaemia	1
Metastatic mammary ca	1
Colon carcinoma	2
Ovarian carcinoma	1
Spinocellular carcinoma*	1

* Immunosuppressed patient due to renal transplant

A recent report has shown that even a smaller tumour increases the risk of metastasis (Mott et al. 2004). Mott and co-workers defined a tumour size of > 0.5 cm as a predictor for poor overall survival. In their study, the statistical significance was established (p = 0.047) but, as they themselves state, it is not highly significant. They attribute greater statistical importance to other markers (invasion into the subcutaneous adipose tissue (p = 0.005), diffuse growth pattern (p = 0.04) and heavy lymphocyte infiltration (p = 0.017)). The 0.5-cm line in poor overall survival seems quite low. It means that practically every single MCC patient should be considered a high-risk patient and should undergo extensive surgical and oncological treatment, thus increasing morbidity in the elderly patient population.

1.2 Treatment and prognosis

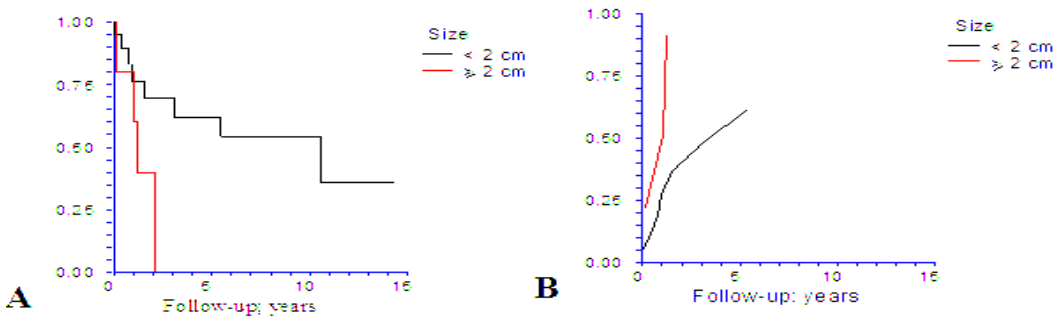
Most of the directly closed excisions were done on small tumours after excision biopsy. In these operations, the margins were from 1 to 2 cm, but we have no exact knowledge of the margins in excision biopsy, as these procedures were usually performed at the primary care centre. In larger operations, the margins were clearer, from 2 to 5 cm.
Only one favourable prognostic factor for OS was established: reconstruction of the defect with split thickness skin graft or local flap (p < 0.02). Obviously, the method of reconstruction reflects

merely the extent of surgical management. It could be postulated that the primary operation is more aggressive when the defect is reconstructed with plastic surgical methods. A simple excision with direct closure can be performed with acceptable wide margins more readily in some areas of the body than in others. Most cases of MCC occur in the head and neck region, where acceptable cosmetic results cannot be achieved without compromising the basis of oncological surgery unless the methods of plastic surgery are employed.

In the literature, margins of 2-5 cm are recommended for better survival (Yiengpruksawan et al. 1991). Applied at the clinical level, the present finding, that is, reconstruction of the defect with a split thickness skin graft or local flap, reinforces the previous recommendations based on epidemiological studies put forward by Yiengpruksawan and co-workers. Some authors do not share this view, however, as larger free margins were found to have no effect on survival (Gillenwater et al. 2001). Additional prognostic factors for poor survival noted here were male sex ($p < 0.05$) and metastatic dissemination at any point during the follow-up ($p < 0.009$).

All events in the course of the disease, that is, re-excisions, local recurrence, re-operations, metastasis and death, were recorded meticulously and examined in a cumulative hazard curve, Figure 4. The curve is very steep in the first two years, indicating that the first years are crucial for disease progression.

Figure 4 Overall survival and cumulative hazard figures from study I. **A** presents the survival of the patients according to the tumour size. **B** shows the cumulative hazard report of 34 MCC patients in the study I, recorded events: local recurrence, re-operations, metastasis and death.



2. DNA copy number changes and metastasis

Different types of chromosomal irregularities have been documented in MCC tumours and cell lines (Van Gele et al. 1998b; Popp et al. 2002; Van Gele et al. 2002). The most common aberrations engage chromosomes 1, 6, 11 and 13. Trisomy of chromosome 6 is a widely considered as recurrent aberration (Larsimont and Verhest 1996; Gancberg et al. 2000). Trisomy of chromosome 1 appears to be typical of MCC (Vortmeyer et al. 1998). In the chromosome 11 a partial trisomy has been documented (Leonard et al. 1993) as well as complete trisomy (Amo-Takyi et al. 1999). In chromosome 13 loss was recognizable (Leonard et al. 1993) in addition to LOH (Leonard and Hayard 1997). So far it seems that high-copy number amplifications for chromosomal subregions are a rare event in MCC (Van Gele et al. 1998a).
In this study, CGH showed several DNA copy number changes in 13 (70%) of the 19 samples. These changes include recurrent gains of chromosomes 1q, 3q, 5p, 8q, 19, and X as well as losses of 3p, 5q, 8p, 10q, 11q, 13q, and 17p, among others. Typically, each sample had several aberrations, ranging from 1 to 12 per sample, average 5.5. This finding is in line with previous studies (Popp et al. 2002). In soft tissue sarcomas tumour progression associated with increase of DNA copy number changes (Popov et al. 2001)

In the present study in 8 (42%) of the tumours, over-representation of all genomic material of chromosome 6 was observed. Furthermore, it was shown that aberrations in chromosome 6 were seen solely in large tumours (size ≥ 2 cm). Five (63%) of those patients developed metastasis in the course of disease. Six of the tumour samples showed gain of DNA sequence copy number affecting either the whole chromosome 1 or different regions of this chromosome.
Although trisomy 1 appears to be characteristic of MCC, its specific role is still unknown. It is thought that it confers proliferative advantages on the tumour cells in MCC (Schlegelberger et al. 1994). In our material 66% of the patients with gains in chromosome 1 progressed to nodal disease.

In a recent molecular study, Vortmeyer and co-workers found that among 10 MCC, seven showed deletions involving chromosome 1p35–36 by LOH analysis (Vortmeyer et al. 1998). The same location has been suspected for genetic changes in pheochromocytoma, neuroblastoma, and (arguably) melanoma. The authors concluded that Merkel cell carcinogenesis shares pathogenetic mechanisms with other neoplasms of neural crest derivation. In this present study, six of the MCC tumour samples showed aberrations in chromosome 1, but none of these were deletions in chromosome 1p35–36.
Furthermore, this study made known some previously not published aberrations such as that 50% of the eight tumours with an increased copy number affecting the whole chromosome 6, had a gain of the whole chromosome 12. This might suggest that the presence of simultaneous gains of

chromosomes 6 and 12 may contribute to the development and progression of MCC. But further studies are needed to confirm this.

One of the interesting results of the current CGH study was the almost complete absence of deletion of chromosome 3. Only one patient presented with deletion of chromosome 3p. This finding is contrary to a previous study that reported LOH on 3p to be a common abnormality in primary MCC (Leonard et al. 1996). On the other hand Popp and co-workers have reported in their study that loss of 3p is rare in MCC (Popp et al. 2002). In their study the loss of 3p correlated with reduced expression of the fragile histidine triad (FHIT) gene, the potential tumour suppressor mapped to 3p14.2. The FHIT gene involved in the pathogenesis of human lung cancers (Oh et al. 2002). It seems that MCC does not share the same pathogenesis route with other neuroendocrine carcinomas and other nonmelanoma skin cancers.

There is a discrepancy between the findings concerning the deletions of chromosome 3. This might be explained by the methodological differences. CGH detects only clonal changes. The aberrations in subclones may balance each other, thus giving normal results. It is also possible the DNA copy number changes were too small to be detected by GCH, whereas, LOH can point out even small, gene specific changes. CGH does not show balanced aberrations, so a sample with specific translocation will appear normal.

In those tumours expanding to metastasis showed more DNA copy number changes than tumours with localised course of disease. A three-fold risk was observed between genetic alterations and poor clinical outcome. Most of these genomic abnormalities (9/13, 69%) were found in large tumours (size ≥2 cm)**.** Of the six tumours that showed no genomic aberrations, five (83%) were small tumours. Although none of those resulted in significant statistical correlation, there was a clear tendency towards it. The small number of samples might explain the statistical correlation, or the lack of it in the present study.

3. Cyclin-A and cell cycle control

Cyclin-A has demonstrated its worth as a prognostic factor in different types of carcinomas and sarcomas (Furihata et al. 1996; Volm et al. 1997; Noguchi et al. 2000; Kyushima et al. 2002). As well as correlating with clinicopathological features, the quantity of cyclin-A expression correlated with OS in oral squamous cell carcinoma (Chen et al. 2003). In soft tissue sarcomas, a correlation has been found between cyclin-A expression and a favourable response to chemotherapy (Huuhtanen et al. 1999). In skin tumours (melanomas), cyclin-A was an independent indicator of a relapse-free period (Florenes et al. 2001). In this study, marked overexpression of cyclin-A was detected but, interestingly, was not statistically confirmed as a prognostic factor. The mean expression of all the tumours was high, 25% (range 5 – 69%). There

was a slight difference in the labelling index between metastatic (30%) and non-metastatic tumours (22%). Even though this study failed to verify a statistical correlation between overexpression of cyclin-A and metastatic potential, there was an undeniable tendency in this direction. The statistical correlation of cyclin-A with local recurrence was not significant (p = 1.0). The labelling index of cyclin-A in MCC is high; it is even higher in samples that expand to metastasis, reflecting the aggressive nature of this disease. Tang and co-workers have presented similar findings for melanomas (Tang et al. 1999).

The association between cell cycle and cancer has become clear in recent years, and dysregulation of cellular proliferation is now regarded as a hallmark of cancer. Dysregulation of the cyclin-CDK-CDKI network has been reported in human and animal tumours in both experimental and clinical studies. Besides cyclin-A, there are other checkpoints in the cell cycle control. Others include for instance the disturbances in apoptosis and cell cycle inhibitors. The apoptotic pathway can be disturbed by oncogene activation (e.g. bcl-2), suppressor gene deactivation (e.g. p53, p21) or mutations (Sherr 1996). The p21WAF1/CIP1 protein is a universal inhibitor of G1 cyclin-dependent kinase and is induced by p53-dependent and -independent pathways. p21 acts as a cell cycle inhibitor, preventing cells from shifting from the G1 to the S phase by blocking cyclin/CDK complex activity. Expression of p21 results in an accumulation of cells in G0/G1, altering cell morphology and differentiation, but not triggering apoptosis (Yang et al. 1995). In an experimental study on mice, Balasubramanian and co-workers demonstrated that, in normal skin, p16 and p21 were not detectable but that in skin tumours, expression of these proteins was significant. Cell cycle deregulation in the G1 phase is a critical event in the course of experimental skin carcinogenesis (Balasubramanian et al. 1998).

DNA damage before S phase results in an increase in p53 concentration, which arrests the cell cycle. p21 mediates p53-induced G1 cell cycle arrest resulting from DNA damage. The p53 tumour-suppressor gene is the most frequent target for genetic alteration cell cycle arrest in G1 and initiation of DNA repair (Hollstein et al. 1991). If repair fails, p53 induces Bax gene apoptosis (el-Deiry et al. 1993). However, if p53 is mutated or loss, cells with damaged DNA continue to proliferate. In MCC p53 expression is mostly negative (Kennedy et al. 1996; Carson et al. 1998). The negativity of p53 may indicate that correction processes after DNA damage in the cell cycle are arrested and apoptosis is not executed. This conclusion is supported by Carson and co-workers in MCC (Carson et al. 2000).

The chromosome locus 9p21 harbours the CDKN2A/ARF tumour suppressor gene, which encodes two cell cycle regulatory proteins, cyclin-dependent kinase 2A (p16 (INK4a) and the alternative reading frame (p14 (ARF)). Cook and co-workers have shown that neither transcript of the CDKN2A locus is the target of deletions on 9p in MCC (Cook et al. 2001). In the current study, only two patients presented with genomic aberrations in chromosome 9; the tumours of

both expanded to metastasis. This present finding seems to be on contradiction with previous studies where the deletions in chromosome 9 are crucial in CDKN2A control.

Although the natural course of MCC is usually defined with metastatic spread, there are some reports in the literature (approximately 10 cases) of spontaneous regression (Connelly et al. 2000). Mori and co-workers have studied the apoptosis in eight cases of MCC (Mori et al. 2001) and found high apoptotic rate in MCC samples. Inoue and co-workers have examined the mechanisms behind the spontaneous regression in seven MCC samples (Inoue et al. 2000). The TUNEL index and number of lymphocytes around the tumour nests was increased in the samples with spontaneous regression, furthermore the majority of the infiltrating lymphocytes were T-cells (Inoue et al. 2000). They concluded that apoptosis and local T-cell mediated immune response might be involved in spontaneous regression of MCC.

4. MCC and Cox-2

Previous studies have shown that overexpression of Cox-2 is a useful prognostic factor in cancers and precancerous lesions of various origin, such as colon carcinoma (Tsujii et al. 1997) and breast cancer (Soslow et al. 2000). The prostaglandins mediate several stages of cancer progression, e.g. cell proliferation and apoptosis, modulation of the immune system and angiogenesis (Hanif et al. 1996 ; Hla et al. 1993). Overexpression of Cox-2 has been shown to correlate with colorectal carcinomas, stimulating the metastatic potential (Tsujii et al. 1997). Tomozawa and co-workers have indeed shown increased haematogenous metastasis following overexpression of Cox-2 in colorectal cancer (Tomozawa et al. 2000). It has been demonstrated that Cox-2 expression is strong in well differentiated tumour cells but that it tends to be weaker in poorly differentiated tumours (Kagoura et al. 2001).

Although the overall expression of Cox-2 was shown to be low, in the present study, over 60% of tumours expanding to metastasis stained positively for Cox-2; in Cox-2 negative cases, the metastasis rate was 42%. A slight tendency for overexpression of Cox-2 was noted in sun-exposed areas of the body (73% in the head and neck region, 75% in the extremities). Only one of the three tumours located in the trunk was positive for Cox-2. With a small sample group like ours, it is, however, impossible to say anything about the statistical significance of overexpression of Cox-2 in sun-exposed areas. In the literature, UVB irradiation exposure has been shown to amplify epidermal Cox-2 expression (Buckman et al. 1998).

With such sound evidence of the metastatic potential of Cox-2 overexpression, it seems only logical to explore the therapeutic possibilities of Cox inhibitors, the non-steroidal anti-inflammatory drugs (NSAIDS), in both cancer prevention and intervention. A growing number of

recent reports discuss the therapeutic effects of Cox inhibitors in chemoprevention (Fischer 2002) and also in tumour regression in experimental animal models (Robertson et al. 1998).

The Cox-2 gene is located at 1q25.2 – q25.3 (Kosaka et al. 1994). In the present study, comparative genomic hybridisation methods did not reveal any genomic aberrations in this particular chromosomal region. Previously Knosel and co-workers confirmed the relationship between Cox-2 and tumour progression and metastasis in colorectal cancer, which could be observed at protein level, reflecting chromosomal gain at locus 1q25.2-q25.3 (Knosel et al. 2004). In the light of the results of the present study, it appears that irregularities in chromosome 1q25 and its relation to Cox-2 are not important in MCC in either tumour progression or metastasis. The staining pattern of Cox-2 in MCC resembles patterns of Cox-2 expression discovered in other neuroendocrine carcinomas, such as SCLC and neuroendocrine carcinoma of the colon (Wolff et al. 1998; Soslow et al. 2000). In the light of the present results, it seems that MCC is not the primary target of NSAIDs in chemoprevention.

5. Tenascin-C and MCC

Staining for Tn-C was positive in 17 (68%) of 25 samples. Of the 11 small (< 2 cm) tumours, 6 (55%) showed negative staining. Staining was positive, however, in 12 (86%) of the 14 large tumours. The staining pattern was located mainly in the invasion borders of the tumour facing the subcutaneous fat. In nine samples, the connective tissue septae within the tumour stained positively for Tn-C. The only statistically significant parameter was Tn-C expression that correlated with large tumour size ($p < 0.035$).

Cells are surrounded by the extracellular matrix (ECM). As well as holding cells and organs together, it serves as a mediator of receptor-induced interaction between the cells. In cell proliferation and morphogenesis, this is done by guiding the growth and differentiation of cells (Adams and Watt 1993).Interactions between the ECM and tumour cells are important in tumour invasion and metastasis. Overexpression of tenascin is believed to facilitate tumour invasion. Both cancer cells and adjacent stromal cells can produce tenascin to co-ordinate the surrounding microenvironment (Sakakura and Kusakabe 1994; Ishihara et al. 1995). The accumulation of Tn-C in the invasion borders of the tumour is a staining pattern that correlates with poorer prognosis (Jahkola et al. 1998). Immunohistochemical detection of Tn-C does not reveal the malignant potential of an individual tumour exactly; rather it shows the capacity of the tumour to invade and the capacity of the cells to proliferate.

The human tenascin gene is located on chromosome 9. Furthermore, in situ hybridisation studies have demonstrated that human tenascin is located at 9q32-q34 (Rocchi et al. 1991). Two of the

MCC samples (12 and 16) in this study showed gains in chromosome 9. Subsequently, both patients expanded to metastasis. In immunohistochemistry analysis, both stained positively for Tn-C (staining intensity + and ++). In these samples, the expression was sizeable and the connective tissue septae were stained.

6. Summary of immunohistochemical analysis

These immunohistochemical analyses revealed several new interesting characteristics of MCC. As expected the tumours with metastatic dissemination also showed enhanced expression in immunohistochemical analysis. In the cyclin-A expression, the difference was not as striking (LI > 20%) 55% of tumours expanding to metastasis and 45% of tumours with no metastastic spread. However, the difference was clearer in Cox-2 and Tn-C expression (63% and 82% respectively in tumours with metastasis). When correlated against the tumour size, in cyclin-A analysis in small sized tumours (< 2 cm) the mean expression was 25% and with large tumours, the mean expression of cyclin-A was 28%. However, in the large tumours 7 (58%) out of 12 tumours showed overexpression of cyclin-A. Cox-2 analysis did not show any differences between small and large tumours. The Tn-C expression was stronger in large tumours than in the small ones with significant statistical correlation. Of the eight Tn-c negative tumours, six (75%) were of small size.

The different combinations of immunohistochemical results were tested statistically. Fischer's exact test showed that these results did not correlate with each other, $p > 0.05$. Therefore, they remain as independent factors for survival.

SUMMARY AND CONCLUSIONS

These studies focused on the search for prognostic factors that would predict the course of MCC. For this purpose, a total of 38 primary MCC samples were reviewed and analysed both immunohistochemically and on a molecular level. The results were analysed statistically to compare them with the clinical outcome of the patients.

1. large tumour size (≥2 cm) was an independent indicator of poor survival whereas, large operation predicted superior survival

2. primary MCC samples showed numerous DNA copy number changes in many chromosomes, that were mostly discovered in large (≥2cm) tumours. Tumours with DNA copy number changes were more likely to disseminate

3. cyclin-A was overexpressed in MCC samples. Correlation, albeit statisically insignificant, was noted between cyclin-A expression and tumour size and with those tumours expanding to metastasis

4. Cox-2 expression in general was low, though most of the samples showed positive staining for it. The Cox-2 inhibition seems not to have role in MCC treatment protocols

5. Tn-C expression correlated statistically with large tumour size, the staining pattern was similar to that discovered in other malignant tumours

The only independent factors predicting the course of disease were found to be large tumour size at presentation and expression of Tn-C. The other immunohistochemical staining results as well as the results revealed by comparative genomic hybridisation showed a strong tendency towards predicting the course of disease. Even though the number of samples in the studies was quite large for MCC, it was apparently too small for significant statistical analysis. Previously unidentified qualities of the tumour were revealed, demonstrating that the course of MCC can be explained with basic scientific methods. Due to the rarity of the carcinoma, clinicians and scientists are often tempted to publish single case studies instead of exploring the true nature of the tumour.

Throughout the studies, two distinctly different patterns of behaviour were emerged. The first pattern, particularly in large tumours, is aggressive and malignant with poor survival. All immunohistochemical analyses (proliferation, invasion and metastasis) showed a tendency for enhanced expression in large tumours and in tumours expanding to metastasis. These findings indicate that the larger the tumour, the greater is its propensity to metastasise. In survival analy-

sis, a line between small and large tumours was established at 2 cm. If the tumour grows beyond this, there is a greater risk for a severe course of disease. The second pattern, mainly in small tumours, has a more benign course with better survival. Therefore, wide surgical excision is recommended for better survival in every case. The risk of developing a metastatic course of disease is twofold in patients with large tumours. An even greater risk was established in patients with tumours carrying chromosome aberrations. The risk of developing metastasis in the presence of genomic imbalances was three-fold. The first two years are crucial for the disease, and treatment and follow-up should therefore be stringent and conducted at regular intervals, preferably in plastic surgery units.

In conclusion, the highly malignant nature of MCC was confirmed in this large series. Large tumour size indicated poor survival, and therefore aggressive surgical treatment is strongly advocated, especially for such patients. This dissertation suggests that factors leading to progression of MCC are of a multifactorial nature and can be traced down to the molecular level. A tendency to poorer prognosis was observed in overexpression of cyclin-A and Cox-2. Tn-C expression correlated with large tumour size. The immunohistochemical analyses performed to reveal these changes are easy to use and affordable in routine pathological practice. On the molecular level, MCCs exhibit multiple DNA copy number changes at many chromosomal locations. Tumours with DNA copy number changes were more likely to metastasise. With the aid of the above prognostic factors every patient can be guided to the right treatment direction, thus avoiding unnecessary procedures and excess morbidity and mortality.

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"Man's perceptions are not bounded by organs of perception: he perceives more than sense (tho ever so acute) can discover."

William Blake

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