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PROGNOSTIC FACTORS OF PRIMARY CUTANEOUS MELANOMA

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Academic Dissertation

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To my family

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LIST OF ORIGINAL PUBLICATIONS

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I Ilmonen S, Asko-Seljavaara S, Kariniemi A-L, Jeskanen L, Pyrhönen S, Muhonen T. Prognosis of primary melanoma. *Scand J Surg* 2002; 91: 166-71.

II Ilmonen S, Hernberg M, Pyrhönen S, Tarkkanen J, Asko-Seljavaara S. Ki-67, Bcl-2 and p53 expression in primary and metastatic melanoma. *Melanoma Res* 2005; 15; 375-81.

III Ilmonen S, Kariniemi A-L, Vlaykova T, Muhonen T, Pyrhönen S, Asko-Seljavaara S. Prognostic value of tumour vascularity in primary melanoma. *Melanoma Res* 1999; 9: 273-8.

IV Ilmonen S, Jahkola T, Turunen JP, Muhonen T, Asko-Seljavaara S. Tenascin-C in primary malignant melanoma of the skin. *Histopathology* 2004; 45: 1-7.

V Ilmonen S, Vaheri A, Asko-Seljavaara S, Carpen O. Ezrin in primary cutaneous melanoma. *Modern Pat* 2005; 18: 503-10.

ABBREVIATIONS

ABC	avidin-biotin-peroxidase complex
AgNOR	silver staining nuclear organiser region
AJCC	American Joint Committee on Cancer
Akt	murine v-akt oncogene homologue
ALCAM	activated leukocyte cell adhesion molecule
APAF-1	apoptotic protease activating factor
ARF	alternative reading frame
Bax	Bcl-2-associated X protein
bcl-2	B-cell lymphoma/leukaemia-2 gene
bFGF	basic fibroblast growth factor
BRAF	v-raf murine sarcoma viral oncogene homologue B1
c-myc	oncogene of the MC29 myelocytomatosis virus
DFS	disease-free survival
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EGF	endothelial growth factor
ERM	ezrin-radixin-moesin
FGF	fibroblast growth factor
gp100	melanocyte differentiation antigen
G0 phase	the zero gap phase of the cell cycle
G1 phase	the first gap phase of the cell cycle
G2 phase	the second gap phase of the cell cycle
HA	hyaluronan
HMB-45	melanocytic cell-specific antibody
IAP	inhibitor of apoptotis protein
ICAM	intercellular adhesion molecule
IGF	insulin-like growth factor
IL	interleukin
INF-α	Interferon-α
Ki-67	nuclear protein expressed in proliferating cells
LDH	lactate dehydrogenase
M phase	mitosis phase of the cell cycle
Mab	monoclonal antibody
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase
MART-1/Melan-A	melanocyte differentiation antigen recognised by T cells 1
Mcl-1	myeloid cell leukemia sequence 1

MC-1R	melanocortin-1 receptor
MCAM	melanoma cell adhesion molecule
MGSA/GRO	melanoma growth stimulatory activity/growth-regulated protein
MIA	melanoma-inhibitory activity
Mib-1	antibody to Ki-67
Mitf	microphtalmia-associated transcription factor
MMP	matrix metalloproteinase
NRAS	neuroblastoma RAS viral (v-ras) oncogene homologue
ns	nonsignificant
OS	overall survival
p	probability
PBS	phosphate-buffered saline
PCNA	proliferation cell nuclear antigen
PDGF	platelet-derived growth factor
PECAM-1/CD-31	platelet/endothelial cell adhesion molecule-1
PET	position emission tomography
PKB	protein kinase B
pT	primary tumour stage according to tumour thickness and level of tumour invasion
PTEN	phosphatase and tensin homologue (mutated in multiple advanced cancers 1)
p75	nerve growth factor receptor
Rb	retinoblastoma gene
RFS	relapse-free survival
RT-PCR	reverse transcriptase polymerase chain reaction
S phase	synthesis phase of the cell cycle
SLN	sentinel lymph node
SLNB	sentinel lymph node biopsy
S100	melanocyte differentiation antigen
Tn-C	tenascin-C
TGF-β	transforming growth factor-β
TNF	tumour necrosis factor
TNM	tumour-node-metastasis
TP53	tumour suppressor gene
UV	ultraviolet
UVR	ultraviolet radiation
VEGF	vascular endothelial growth factor

ABSTRACT

A proportion of patients with early-stage cutaneous melanoma will eventually die of the metastatic disease. Prognostic factors are needed to identify this subgroup so that they can be allocated for more effective adjuvant treatments and surveillance. Biological prognostic factors may also serve as future treatment targets. The most common clinicopathological prognostic markers, *i.e.* tumour thickness (Breslow classification) and level of tumour invasion (Clark classification), were evaluated in a clinical retrospective series of 298 primary melanoma patients. In addition, tumour proliferation (Ki-67), Bcl-2, p53 and tumour vascularity (CD-31), were measured immunohistochemically in a subset of tumours and the results were related to disease outcome. In the search for new biological prognostic markers, expressions of the proteins tenascin-C (Tn-C) and ezrin were measured and related to the course of the disease. Further, in 18 patients the first metastatic tissue specimen was analysed for Ki-67, Bcl-2 and p53 and in 12 patients for ezrin. The findings were then compared with the corresponding immunohistochemistry of primary tumours.

The majority of the patients presented with localised stage I or II tumours at diagnosis; the overall survival rate at the 9.5-year follow-up was 66.8%. The most important factors determining the prognosis were tumour thickness, level of tumour invasion and stage at diagnosis. Other significant adverse prognostic factors were tumour ulceration, tumour location on trunk and older age of patients. The width of the surgical resection margins had no effect on survival in any Breslow category. Strong immunoreactivity of Bcl-2 in primary melanomas was associated with adverse clinicopathological features such as male gender, older age and tumour ulceration and was associated with an adverse prognosis in intermediate thickness melanomas. Strong p53 immunoreactivity was an adverse prognostic factor for disease-free survival, whereas expression of Ki-67 had no prognostic value. In melanoma metastases, expressions of Bcl-2 and p53 were lower than in their primary counterparts, Ki-67 showing no trends during disease progression. Patients with high tumour vascularisation showed a trend towards better overall survival. Expression of Tn-C had no correlation with tumour staging. Positive immunoreactivity for Tn-C in invasive regions of the tumour and intracytoplasmically in melanoma islets was important in the progression of primary melanoma to metastatic disease but did not influence the prognosis significantly thereafter. Expression of ezrin in primary melanoma was associated with tumour proliferation and tumour growth but was not a prognostic factor. Expression of ezrin was, in general, stronger in melanoma metastases than in primary tumours.

In conclusion, the most important prognostic markers in this patient population with mainly localised primary melanomas were the stage of the tumour at diagnosis, and the thickness and invasion level of the tumour. Strong p53 expression was as-

sociated with adverse disease-free survival, and strong Bcl-2 expression with other adverse clinicopathological features; Ki-67 expression was not a prognostic factor. High tumour vascularisation was associated with a better prognosis, but was not an independent prognostic factor. Absence of Tn-C in tumours was related to more benign disease behaviour and a lower risk of developing metastases. Expression of ezrin correlated with tumour proliferation (Ki-67), thickness and level of invasion, suggesting an association between ezrin expression and tumour progression, but did not reach prognostic significance in survival analysis.

INTRODUCTION

Melanoma has long been one of the fastest increasing malignancies in most Caucasian populations. In Finland, 716 new cases of primary melanoma and 155 deaths from melanoma were documented in 2003 (Finnish Cancer Registry 2005). Melanoma is most likely surgically cured if the tumour is thin (≤ 1.0 mm), superficial and non-ulcerative, the 10-year survival rate being 88%. The thicker the tumour, the worse is the prognosis. The 10-year survival rate for patients with a melanoma 1.01 – 4.0 mm thick is 51 – 79%, but for those with melanomas over 4.0 mm thick the rate is 32 – 54%. If clinical lymph nodes are involved at diagnosis, the 10-year survival rate is 18 – 48% (Balch et al. 2001a). The major cause of death from melanoma is distant metastasis. Melanoma has traditionally been considered resistant to chemotherapy and radiation, and metastatic disease has a poor prognosis, with a median survival rate of 6 months (Manola et al. 2000, Unger et al. 2001). The difficult problem facing us is to discover an oncological treatment that can cure systemic disease. As long as early surgery is the only reliable method to arrest disease progression, emphasis has to be on early accurate diagnostics, including the evaluation of prognostic parameters.

The clinical behaviour of individual melanoma patients is unpredictable and presents clinicians with a major challenge. Previous studies have reported that among superficial melanomas (Breslow score ‹ 1.0 mm) some tumours behave aggressively and metastasise early whereas some patients with thick melanomas do surprisingly well and survive for longer than expected (Spatz et al. 1998, Kalady et al. 2003). Melanoma has a multifactorial aetiology and much of its genetic and immunological background is still unknown. Research on melanoma biology including identification of the factors crucial to melanoma development and progression, may help us to recognise the high-risk patients who might benefit from more effective treatments and follow-up. Another hope is that we could develop successful targeted treatments. Much research has focused on finding prognostic markers for primary melanoma. So far tumour thickness (Breslow classification) has remained the most important tumour-related prognostic factor for localised primary melanoma. The second strongest predictor of disease outcome is tumour ulceration; the level of tumour invasion (Clark classification) is a significant factor only in thin melanomas (Balch et al. 2001a). Recent studies suggest that the presence or absence of dissemination of melanoma cells to the sentinel lymph node (SLN), the first lymph node in the lymph basin draining from the tumour, is the most important factor in the prediction of disease outcome (Morton et al. 1992, Balch et al. 2004).

This thesis is based on a Finnish patient population (n = 298) treated for primary melanoma during 1988-1991 at Töölö Hospital, Helsinki University Hospital, with a median follow-up time of 9.5 years. The most important clinicopathological prog-

nostic markers and the survival of patients are presented. In our search for biological prognostic factors we conducted immunohistochemical studies of tumour proliferation (Ki-67), the antiapoptotic protein Bcl-2, tumour-suppressor gene product p53, and tumour vascularity (CD-31). All these results are compared with the results of previous reports. In addition, the expression patterns of two new biomarkers, tenascin-C and ezrin, are presented and correlated with classical tumour markers and the clinical outcome of the patients. Understanding the clinical significance of biological parameters of cutaneous melanoma may help us to select high-risk patients who could profit from more effective treatments.

REVIEW OF THE LITERATURE

1. Primary melanoma

1.1. Epidemiology

Cutaneous melanoma, a malignant lesion arising from the melanocytes, is less common than basal or squamous cell carcinoma of the skin but it is much more fatal (de Vries and Coebergh 2004). Between 1940 and the mid- to late-1980s, the incidence of invasive cutaneous melanoma increased more rapidly than any other cancer in populations of predominantly Caucasian origin, the annual rate of increase being 3–7% (Diepgen and Mahler 2002). However, the incidence rates for melanoma show substantial variation worldwide (Lens and Dawes 2004). Currently, the highest rates have been reported from Australia, where the age-standardised rate for men is 39/100 000 and for women 30/100 000. The lowest rates have been reported from China, 0.2/100 000 for both men and women (Ferlay et al. 2004).

In Northern Europe, where the incidence rates rose sharply during the 1980s, the rates seem to have been levelling off since the mid-1990s, especially in younger age groups. Primary prevention, *i.e*. less sun exposure, is thought to have been an important factor contributing to these shifts (de Vries et al. 2003, Lindholm et al. 2004). In contrast, in Southern and Eastern Europe, the rates are continuing to rise steeply in all age categories (Severi et al. 2000). Over the last decades, increases in incidence have mainly been reported for thin melanomas, whereas the rate for thick melanomas seems to have been fairly stable (Lipsker et al. 1999, Murray et al. 2005).

Currently, the rising trend in mortality appears to have come to a halt, or even fallen, in the young and middle-aged in many populations with high melanoma incidence rates, such as Australia and the United States and countries in North-Western Europe (La Vecchia et al. 1999, de Vries et al. 2003). One possible reason for this development is earlier diagnosis. However, there has been no sign of a downward trend in mortality in countries with lower incidence rates, *e.g*. in Southern Europe (Severi et al. 2000, de Vries and Coebergh 2004). In addition, high mortality rates have persisted among older men (Streetly and Markowe 1995, Buettner et al. 2005).

In Finland, the incidence of melanoma rose continuously until the late 1980s, when it started to level off. In 2003, the incidence of melanoma was 9.3/100 000 (362 new cases) among men, and 7.9/100 000 (354 new cases) among women (Finnish Cancer Registry 2005). According to the Finnish Cancer Registry, primary melanoma was the twelfth most common cancer among both men and women in Finland in 2003. Since 1953, the mortality rates have consistently been higher in men than in women, reaching a peak of 2.6/100 000 in 1995, after which the rates declined slight-

ly, being down to 2.3/100 000 in 2003. The mortality rates for women have remained fairly steady since the early 1970s, and in 2003 stood at 0.9/100 000 (Finnish Cancer Registry 2005).

1.2. Risk factors for melanoma

The precise cause of melanoma is unknown. The likelihood of cutaneous melanoma developing in a person depends on his or her constitutional predisposition, *i.e.* the genotypic and phenotypic characteristics, and subsequent exposure to environmental risk factors (Armstrong 2004).

1.2.1. Environmental factors

Melanoma is primarily a disease of Caucasians. Among them, exposure to ultraviolet radiation (UVR) is the major environmental risk factor for melanoma, and is thought to be responsible for the increasing incidence of the disease (Thompson et al. 2005). Worldwide, the incidence of melanoma in Caucasians generally correlates inversely with latitude *i.e*. rates are generally higher close to the equator and become progressively lower in areas nearer to the poles (Ferlay et al. 2004). The association between UVR and melanoma is ambiguous, with differences in risk depending on the dose, the way the dose is delivered (intermittent or chronic) and the patient´s age (de Vries and Coebergh 2004). Approximately 95% of solar UVR reaching the ground is UVA (wavelength 320 to 400 nm), which penetrates deeper into the dermis while the remaining 5% is UVB (wavelength 290 to 319 nm), which is the radiation mainly responsible for erythematous response in the skin. UVB and probably also UVA are risk factors that can induce mutations in skin cells and suppress immune reactions (Marks 1999). Intermittent, unaccustomed exposure to UVR causing sunburns, especially during childhood and adolescence, has been postulated as the main risk factor for the development of melanomas (Desmond et al. 2003, de Vries and Coebergh 2004). Without repair of the damage, mutations in DNA can eventually result in the formation of melanoma, though the exact mechanisms of these mutations and events in the cells are still unknown (Owens and Watt 2003).

Commonly used sunscreens reduce the transmission of UVB to the skin, thus providing protection against the development of erythema but not the immunosuppression caused by UVA (Kelly et al. 2003, Poon et al. 2003). To date there is no evidence of the protective role of sunscreen use against the development of melanoma (Diffey 2005).

1.2.2. Genes involved in melanoma susceptibility

Melanoma has a heterogeneous genetic aetiology. On the basis of population studies, it is suggested that approximately 5–12% of patients with melanoma have a family history of melanoma in one or more first-degree relatives (Goldstein and Tucker 2001). Some of these patients have inherited a mutation in highly penetrant susceptibility genes that are associated with an increased risk of melanoma. Current genetic studies point to two such genes involved in cell cycle regulation: *CDKN2A* (Cyclin-Dependent Kinase Inhibitor), which is located on chromosome 9 (9p21) (Cannon-Albright et al. 1992), and *CDK4* (Cyclin-Dependent Kinase), which is located on chromosome 12 (12q13) (Zuo et al. 1996). Inactivating mutations of *CDKN2A* confer a 50–90% risk of melanoma on carriers by the age of 80 years (Bishop et al. 2002). Several families have been found to have mutations in *CDK4* (Bishop et al. 2002). Penetrance of *CDKN2A* mutations is influenced by environmental or genetic factors, and it varies among populations with different melanoma incidence rates. The prevalence of *CDKN2A* mutation carriers is less than 1% in high-incidence melanoma populations. Moreover, uncertainties still exist regarding genotype/phenotype correlations of *CDKN2A* mutations. It is therefore recommended that predictive genetic testing for melanoma is carried out only in the context of clinical research (Kefford et al. 2002). *CDKN2A* aberrations are found in 20% of sporadic melanomas (Bishop et al. 2002). A locus on chromosome 1p22 is strongly linked with melanoma susceptibility but the gene responsible has not yet been identified (Gillanders et al. 2003).

CDKN2A encodes two tumour suppressor proteins, p16 and $p14^{ARF}$. p16 prevents entry of the cell into the cell cycle by inhibiting CDK4 phosphorylation of the retinoblastoma protein (Serrano et al. 1993). $p14^{ARF}$ is induced in response to hyperproliferative stimuli and is believed to induce p53-dependent cell cycle arrest or apoptosis (Sherr and Weber 2000). Mutations in *CDK4*, in turn, render the protein kinase CDK4 resistant to p16 and are functionally equivalent to p16 loss (Soufir et al. 1998).

Variants in the gene of the Melanocortin-1 receptor (*MC1R)* have been correlated with an increase in melanoma in the general population and also in families with *CDKN2A* mutation. *MC1R* is activated upon hormonal stimulation and induces a production switch from pheomelanin to eumelanin. Loss-of-function mutations accompany a switch from eumelanin to pheomelanin production and are associated with red hair and fair skin (Valderve et al. 1995). *MC1R* gene is highly polymorphic, with more than 30 variants; individuals with several of these variants have a 2–4-fold increase in melanoma risk (Valderve et al. 1996).

Other important genes involved in melanoma are *NRAS* and *BRAF* genes. Mutations in *BRAF* are the most common in human melanoma, occurring in roughly 80% of cultured tumours (Davies et al. 2002), whilst mutations in *NRAS* are identified in 15% of sporadic melanomas, and are associated with sun-exposed sites (van Elsas et al. 1996). Mutations in these genes cause activation of the Ras-Raf-MAPK pathway, a cascade involved in intracellular signalling, and result in an increase in

autocrine growth factors that can promote tumorigenesis. The activated Ras-Raf-MAPK pathway can also lead to changes in gene transcription and resistance to apoptosis (Collisson et al. 2003).

Among the many other genes under active investigation is *APAF-1*, the apoptotic protease activating factor that plays a central role in mitochondria-dependent apoptosis. Loss of Apaf-1 expression is encountered in 40% of sporadic melanomas (Soengas et al. 2001). *PTEN* (phosphatase and tensin homologue) is a tumour suppressor gene (located at 10q23.3) that is involved in cell proliferation, survival, adhesion and migration. Loss of chromosome 10q has been detected in 30–50% of sporadic melanomas with an association of poor clinical outcome (Herbst et al. 1994). The tumour suppressor gene *TP53* and its protein product, p53, are involved in cell cycle regulation. Normal p53 inhibits growth through activation of cell cycle arrest and apoptosis in response to DNA damage. Mutations in *TP53* are rare in melanoma (Hussein et al. 2003).

1.2.3. Chromosomal changes

Melanoma chromosomal pathogenesis is characterised not by specific structural abnormalities but rather by widespread aneuploidy (Casorzo et al. 2005). The chromosomes most frequently involved in structural rearrangements in melanoma are 1, 6, 7, 9, and 11. Studies comparing the chromosomal status in primary and metastatic tumours of the same patient illustrate that losses of 9p and 10q are early events in tumorigenesis, whereas loss of 11q and duplication of chromosome 7 appear to be later events (Albino et al. 1993). Few prognostic studies exist. Nevertheless, patients with metastatic melanoma due to structural abnormalities of chromosomes 7 and 11 have had shorter survival times (Trent et al. 1990).

1.2.4. Host factors

Multiple (› 100) benign nevi are the most common clinical risk factors for melanoma (Berwick and Halpern 1997). Other minor risk factors for whites are blonde or red hair, the presence of freckles, and a fair skin type that is unable to tan. The risk of melanoma is increased in individuals with immunosuppression or with a history of blistering sunburn (Thompson et al. 2005). Individuals with large congenital melanocytic nevi are also at increased risk (relative risk of 101) for developing melanoma; cutaneous and/or extracutaneous. Individuals at greatest risk are those with very large, ≥50 cm congenital melanocytic nevi (Bittencourt et al. 2000).

Dysplastic nevus syndrome is an important clinical risk factor for melanoma. An individual with this syndrome has 50 to 100 clinical atypical nevi on the upper trunk and limbs, some of them showing variability of size, outline and colour. Melanocytic dysplasia is seen in histologically atypical nevi. An individual with dysplastic nevus syndrome has a relative risk of approximately 11 for developing melanoma (Armstong 2004). The genetic background of this syndrome is still unknown.

A melanoma patient has a relative risk of 8.5 for developing a second melanoma (Tucker et al. 1985). A strong family-history (≥ three first-degree relatives affected) increases an individual´s relative risk of developing melanoma to 35–70 (Goldstein and Tucker 1995). Previous non-melanoma skin cancer also increases the relative risk of melanoma to 2.9 (Bower et al. 2000).

Xeroderma pigmentosum is a rare, autosomal recessive genetic disorder characterised by an inability to repair DNA damaged by UVR. Patients with xeroderma pigmentosum have a relative risk of more than 1000 for developing melanoma (Green et al. 2002), and almost 40% of reported melanomas among such patients occurred in children aged 12 years or younger (Lynch et al. 1967).

1.3. Diagnosis

Primary cutaneous melanoma may develop in precursor melanocytic nevi (common acquired, congenital and atypical/dysplastic types) but more than 50% of cases are believed to arise *de novo*, without a pre-existing pigmented lesion (Tucker et al. 2002). The following clinical signs are suspicious for melanoma, especially the superficially spreading type: asymmetry, border irregularity, colour variegation and a diameter exceeding 6 mm. Even more important are changes to the lesion with respect to size, shape, shades of colour and surface features that may be signs of malignancy. Itching and tenderness of a lesion may indicate a malignant process (Abbasi et al. 2004). In clinical monitoring of patients with dysplastic nevus syndrome, total body photographs and dermoscopy can be used to detect new and changing nevi.

The clinical morphological spectrum of melanoma is broad and diverse, and includes non-pigmented tumours, which have a reported incidence of 1.6–10% (Thompson et al. 2005). If any suspicion of melanoma arises, the lesion should be submitted to histopathological analysis without delay. The standard procedure is limited but complete excision of the lesion including a narrow rim (2 mm) of normal skin. The diagnosis of melanoma depends on the results of the histological examination, no reliable marker having yet been found to replace the histological examination in melanoma diagnosis. Nevertheless, immunohistochemical analyses using antibodies to melanocyte differentiation antigens such as tyrosinase, gp100, Mart-1/Melan-A, MCAM and Mift are performed in histologically difficult cases such as amelanotic melanoma (Mangini et al. 2002). In differential diagnostics of the rare subgroup of desmoplastic neurotropic melanoma, the immunohistochemical analysis of p75 is used in conjunction with other markers (S-100, tyrosinase, HMB-45) (Kanik et al. 1996).

1.4. Staging

In 2002, the American Joint Committee on Cancer (AJCC) introduced a new, evidence-based melanoma staging system to replace the previous one, which dated from 1997 (Tables 1 and 2) (Balch et al. 2004). The new staging system incorporates several major changes: (1) tumour (T) category thresholds of melanoma thickness are defined as 1.0, 2.0 and 4.0 mm. Tumour thickness is now regarded as the primary determinant of tumour staging; (2) the Clark level of invasion is only used when defining subcategories of T1 (thin ≤ 1.0 mm) melanomas, not for thicker melanomas (*i.e.* T2, T3 or T4); (3) ulceration has been added to the description of the primary tumour and is defined as the absence of intact epidermis overlying a major portion of the primary melanoma on the basis of microscopic examination of the epidermis (Balch et al. 2001a); (4) clinical satellites, microsatellites and in-transit metastases have similar prognostic implications, are categorised as nodal (N) disease, and are placed in the regional stage III disease classification; (5) the size of the lymph node as a prognostic factor has been eliminated and replaced by the number of positive nodes; this is regarded as the primary criterion. Pathological analysis of the tumour burden between micrometastases and macrometastases has been added to staging; and (6) the presence of an elevated serum level of lactate dehydrogenase (LDH) is used to define the metastasis (M) category.

Clinical stages I and II are confined to patients with localised melanoma who have no evidence of metastases on the basis of clinical, radiological and/or laboratory examination. Stage III patients are those with a regional disease showing clinical or radiological evidence of regional micro- or macrometastases in either the regional lymph nodes, satellites or in-transit intralymphatic vessels. Stage IV melanoma patients have metastases at a distant site.

Table 1
Melanoma TNM classification (AJCC 2002)

T classification	**Tumour thickness (mm)**	**Ulceration status**
T1	≤ 1.0	a=without ulceration and Clark level II/III b=with ulceration or Clark level IV/V
T2	1.01 – 2.0	a,b
T3	2.01 – 4.0	a,b
T4	› 4.0	a,b
N classification	**Number of metastatic nodes**	**Nodal metastasis mass**
N1	1	a=micrometastasis* b=macrometastasis**
N2	2–3	a,b, c=satellite(s)/in-transit metastasis(es) *without* metastatic nodes
N3	≥ 4, or matted nodes, or in-transit met(s)/satellite(s) *with* metastatic node(s)	
M classification	**Site**	**Serum LDH**
M1a	distant skin, subcutaneous or nodal metastasis	normal
M1b	lung metastasis	normal
M1c	all other visceral metastases, any distant metastasis	elevated

*Micrometastases are diagnosed after sentinel or elective lymphadenectomy.

** Macrometastases are defined as clinically detectable nodal metastases confirmed by therapeutic lymphadenectomy or when nodal metastasis exhibits gross extracapsular extension.

Table 2
Stage groupings for cutaneous melanoma

	Clinical staging*			Pathological staging**		
	T	**N**	**M**	**T**	**N**	**M**
0	Tis	N0	M0	Tis	N0	M0
IA	T1a	N0	M0	T1a	N0	M0
IB	T1b	N0	M0	T1b	N0	M0
	T2a	N0	M0	T2a	N0	M0
IIA	T2b	N0	M0	T2b	N0	M0
	T3a	N0	M0	T3a	N0	M0
IIB	T3b	N0	M0	T3b	N0	M0
	T4a	N0	M0	T4a	N0	M0
IIC	T4b	N0	M0	T4b	N0	M0
III	any T	N1 N2 N3	M0			
IIIA				T1-4a	N1a	M0
				T1-4a	N2a	M0
IIIB				T1-4b	N1a	M0
				T1-4b	N2a	M0
				T1-4a	N1b	M0
				T1-4a	N2b	M0
				T1-4a/b	N2c	M0
IIIC				T1-4b	N1b	M0
				T1-4b	N2b	M0
				any T	N3	M0
IV	any T	any N	any M	any T	any N	any M

*Clinical staging includes microstaging of the primary melanoma and clinical/radiological evaluation for metastases. By convention, it should be used after complete excision of the primary melanoma with clinical assessment for regional and distant metastases.

**Pathological staging includes microstaging of the primary melanoma and pathological information about regional lymph nodes after partial or complete lymphadenectomy. Pathological stage 0 or stage 1A patients are the exception; they do not require pathological evaluation of their lymph nodes.

1.5. Prognosis

The single best indicator of the prognosis for patients with melanoma is the stage at their first clinical presentation (Balch et al. 2001b). The patients at greatest risk include those with primary tumours › 4 mm thick and those with lymph node involvement. For *in situ* melanomas complete excision is curative. The overall prognosis for patients with localised melanoma is considered rather good. On the basis of the AJCC Melanoma Staging Database, which consists of 17 600 patients (Balch et al. 2001b), the 10-year overall survival rates for patients with stage I (primary tumour ≤ 1.0 mm) tumours was 85% and for those with stage II (primary tumour › 1 mm) tumours 55%. In stage I and II localised melanomas, tumour thickness and ulceration were the two most powerful prognostic factors. In thin melanomas (≤ 1.0 mm), the level of invasion was more predictive of survival outcome than was tumour ulceration. In contrast, in melanomas › 1.0 mm thick, tumour ulceration was more predictive than was level of tumour invasion. Other statistically significant factors were the patient´s age and gender and the site of primary melanoma (extremity vs axial). In stage III melanomas, there were three significant prognostic factors in multivariate analysis: the number of metastatic nodes, the tumour burden at the time of staging (*e.g.* microscopic vs macroscopic) and the presence or absence of ulceration of the primary melanoma. The prognosis among stage III group patients differed markedly, ranging from 69% of the 5-year survival rate for patients with nonulcerated melanomas (regardless of thickness) and a single occult nodal metastasis to 13% of the survival rate for patients with ulcerated melanomas (regardless of thickness) with four or more clinically apparent nodal metastases (Balch et al. 2001b). The median duration of survival for the stage IV group of patients was 6–7.5 months from the time that distant metastasis was documented, whereas the 5-year survival rate was less than 10%. In stage IV melanomas, the anatomical site of the distant metastases was the most significant prognostic factor, the survival rate being higher for patients with nonvisceral metastases (*i.e.* skin, subcutaneous tissue and distant lymph nodes) than for those with visceral metastases (Balch et al. 2001b).

The histological status of the sentinel lymph node (SLN) has been considered an important predictor of disease-free and overall survival in many recent studies, Table 3.

Table 3

Studies on sentinel lymph node biopsy and prognosis

Study	SLN negative patients	SLN positive patients
Statius Muller et al. 2001 (n = 263)		
5-year DFS rate	91%	49%
Doting et al. 2002 (n = 197)		
3-year DFS rate	83%	66%
3-year OS rate	92%	73%
Yee et al. 2005 (n = 836)		
5-year OS rate	90%	56%
Leong et al. 2005 (n = 362)		
5-year DFS rate	69%	38%
5-year OS rate	69%	60%

DFS= disease-free survival
OS= overall survival

1.6. Clinical and histopathological prognostic factors

The anatomical site of a primary tumour has proved to be an independent prognostic factor in many studies (Sondegaard and Schou 1985, Thorn et al. 1994, Balch et al. 2001b). Patients with lesions on the extremities, with the exception of the hands and feet, have a better prognosis than those with lesions on the trunk, head or neck. More specifically, the prognosis is particularly poor for patients with melanomas on the scalp, hands or feet (Balch et al. 2001b, Rogers and Braun 2002). However, contradictory results demonstrated that the primary location of the tumour in acral parts of the body was not an independent prognostic factor when tumour thickness and ulceration were considered using multivariate analysis (Wells et al. 1992). Neither was any prognostic significance found when the tumour was located in the scalp, neck, upper back or outer arm (Evans et al. 1994). Data on the prognostic role of gender in melanoma are contradictory. Some investigators demonstrate that female gender is an independent prognostic factor regardless of tumour location, thickness or ulceration (Stidham et al. 1994, Balch et al. 2001b); others emphasise the difference in the presentation of melanoma in women, who tend to be associated with thin, nonulcerated tumours on extremities (Greenstein and Rogers 1991, Vossaert et al. 1992, Thorn et al. 1994). The age of the patient is an independent

prognostic factor for melanoma, higher age correlating with poorer survival regardless of tumour thickness or ulceration (Cohen et al. 1987, Austin et al. 1994, Balch et al. 2001b).

In 1970, Breslow introduced the concept that prognosis correlated with tumour thickness, as measured in millimetres from the granular cell layer to the deepest part of the melanoma in a histological sample (Breslow 1970). Since then, numerous studies have established tumour thickness as the most reliable tumour-related prognostic factor for localised primary melanomas (Balch et al 2001b, Nagore et al. 2005). According to a recent study, the 10-year survival rate by tumour thickness is as follows: ≤ 1 mm 91%, 1.01–2.0 mm 78%, 2.01–4.0 mm 62%, and › 4.0 mm 38% (Carlson et al. 2003). Clark´s classification, developed by Clark and coworkers in 1969, determines the anatomical involvement of a tumour within the cutaneous and subcutaneous structures. The levels are as follows: level I – intraepidermal growth with intact basement membrane, level II – invasion of the papillary dermis, level III – tumour involvement filling the papillary dermis and involvement of the junction between the papillary and reticular dermis, level IV – invasion of the tumour into the reticular dermis, and level V – invasion of tumour cells into subcutaneous fat (Clark et al. 1969). In the current staging system, Clark´s classification is considered to have prognostic value only in T1 melanomas. Tumour ulceration is an adverse prognostic indicator in stage I, II and III melanomas, but factors contributing to its formation are still unclear (Balch et al. 2001b). Among histopathological subtypes, the most common are superficially spreading melanoma and nodular melanoma, which constitute 85% of tumours, the remaining 15% consisting of lentigo maligna, acral lentigious and desmoplastic melanomas. In general, there are no significant prognostic differences between the subgroups; rather they reflect inherent thickness differences (MacKie 2000). For instance, nodular melanoma is, by definition, thicker than superficially spreading melanoma, and the prognosis of lentigo maligna melanoma is no better than that of other tumour types when stratified by tumour thickness (Koh et al. 1984, Kopf et al.1987).

Many studies have identified vascular involvement of melanoma cells into the lumen of the vessel as an adverse prognostic factor (Straume and Akslen 1996, Kashani-Sabet et al. 2001). Microsatellites are discrete nests of tumour cells separated from the main body of the tumour. They occur infrequently in thin melanomas but their incidence increases in thick melanomas. The presence of microsatellites clearly predicts locoregional relapse and a few studies find a significant correlation between the appearance of microsatellites and a decreased disease-free survival rate (Day et al. 1981, Harrist et al. 1984, Shaikh et al. 2005). The mitotic rate of an invasive tumour is defined as the number of mitotic figures observed per square millimetre. The majority of large studies confirm the mitotic rate as an important marker of disease outcome in melanoma (Retsas et al. 2002, Azzola et al. 2003). Regression in primary melanoma is a histological reflection of host immune interaction with malignant cells, and is

encountered most often in thin tumours. Some authors have suggested that regression is a feature of thin metastasising lesions (Ronan et al. 1987, Mansson-Brahme et al. 1994, Cook et al. 2002), but others have not attributed any significant role to regression in explaining metastasis in thin melanomas (McGovern et al. 1983, Cooper et al. 1985). The presence of lymphocytes infiltrating the base of the tumour during the vertical growth phase is a sign of an immune reaction to the melanoma cells by a host. The prognostic importance of the lymphocytes is an unsettled issue, some studies correlating their presence with a better prognosis (Sondergaard and Schou 1993, Clemente et al. 1996), others denying this (Thorn et al. 1994, Kopf et al. 1987).

1.7. Prognostic serum markers for melanoma

The serum markers currently available for melanoma have only limited clinical use. Among those most widely used are lactate dehydrogenase (LDH), S-100β and melanoma inhibitory activity (MIA). As close correlations exist between their serum concentrations and tumour load, serum markers are not suitable for screening or for the diagnosis of primary melanomas (Ugurel 2005). Patients with distant metastases from melanoma who present with elevated serum levels of LDH, S-100β and MIA have poorer overall survival than do patients whose serum concentrations are within normal ranges (Vuoristo et al. 2000, Balch et al. 2001a and b, Ugurel 2005).

Several studies have focused on the use of reverse transcriptase polymerase chain reaction (RT-PCR) technology to detect melanin synthesis enzymes, most commonly tyrosinase and Mart-1, in melanoma cells circulating in the peripheral blood (Sarantou et al. 1997, Li et al. 2002). In a study comprising stages II and III clinically free melanoma patients, determination of RT-PCR for tyrosinase and Mart-1 in the peripheral blood did not have a significant impact on their prognosis; however, elevated serum levels of S-100β and MIA detected during follow-up examinations were significantly associated with decreased recurrence-free survival rates (Garbe et al. 2003).

1.8. Treatment of primary melanoma

1.8.1. Surgery

Surgery is the principal treatment method for primary melanoma. Current recommendations for excision margins of the primary tumour are based on prospective randomised studies of the World Heath Organization Melanoma Group (Veronesi et al. 1988), the Intergroup Melanoma Surgical Program (Balch et al. 1993), the Swedish Melanoma Study Group (Cohn-Cedermark et al. 2000), the French Group for Research on Malignant Melanoma (Khayat et al. 2003) and the British collaborative trial (Thomas et al. 2004).

For melanoma in situ, an excision margin of 0.5-1 cm is sufficient. In invasive melanomas ≤ 1 mm thick, a margin of 1 cm is adequate. In melanomas 1.01–2.0 mm thick, the margins should be 1-2 cm; in melanomas 2.01–4.0 mm thick, the margins should be 2 cm; and in melanomas › 4 mm thick, margins of at least 2 cm are sufficient. No randomised studies have directly compared 1-cm and 2-cm margins for melanomas 1–2 mm thick. Consensus recommends, however, a 2-cm margin whenever it is anatomically feasible and a 1-cm margin in anatomically restricted areas. To date, only the British collaborative trial has evaluated excision margins for melanomas › 4 mm thick. The conclusion of this trial was that the excision margin for melanomas of this thickness should be 3 cm. Until more results are available from ongoing trials, the recommendation is for margins of at least 2 cm for › 4 mm melanomas (Thompson et al. 2005).

The most recent methods for treating primary melanoma are biopsy and histopathological analysis of the sentinel lymph node (SLN). The concept of SLN is that the first set of lymph nodes receives drainage and cancer cells from the primary tumour site (Morton et al. 1992). According to the AJCC Melanoma Committee recommendation, all melanoma patients with clinically node-negative regional lymph nodes who are considered for entry into surgical and adjuvant therapy clinical trials should have pathological staging with sentinel lymphadenectomy to ensure prognostic homogeneity (Balch et al. 2001a). In clinical practice, sentinel lymph node biopsy (SLNB) is recommended for all melanoma patients with tumours ≥ 1 mm thick or with invasion level IV or V according to Clark´s classification or with ulceration regardless of Breslow´s classification (Testori and Mozzillo 2002). Patients with micrometastases in SLN have undergone reoperation involving dissection of the complete regional node field. In positive SLN cases, however, there has been no evidence of metastatic disease in non-sentinel nodes in 87% of patients. A new multicentre trial has been launched to examine the necessity of complete node dissection for positive SLN cases (Morton et al. 2005). Knowledge of SLN status allows more accurate staging and provides a more reliable estimate of prognosis. These benefits are important not only for patients themselves, but also for stratification in adjuvant therapy trials. The therapeutical results of sentinel lymph node biopsy concerning overall survival are still awaited with longer follow-up of multicentre trial (Morton et al. 2005).

Clinically evident metastatic disease in regional lymph nodes should be treated surgically, by complete regional node dissection, since 13–59% of such patients will not develop further metastatic disease (Balch et al. 2001b). Local recurrences and in-transit metastases are most effectively treated by surgical excision. Careful consideration should be given to the choice of therapy for patients with numerous or large lesions, and for those with concurrent extraregional disease. Redissection of the nodal basin is recommended for recurrent regional lymph node metastases (Balch et al. 2001b, Thompson et al. 2005).

Distant metastases are most common in subcutaneous tissue, liver, lung and brain (Balch et al. 2001b). The patients most likely to benefit from resection of distant

metastases are those with a relatively small number of lesions (*e.g.* one to four) confined to the subcutaneous, nonregional lymph nodes, lung or brain, and a disease-free interval of 1 – 2 years since resection of the primary lesion (Karakousis 1996). The excision of brain metastases has been shown to improve the survival of patients (Cattell et al. 2002). However, the resection of isolated pulmonary metastases or subcutaneous recurrences is not generally considered curative though it may result in a significantly prolonged disease-free interval (Wong et al. 1993, Karakousis 1996). No randomised clinical trials have been conducted on the effectiveness of the surgery of distant metastases.

1.8.2. Radiotherapy

Melanoma has traditionally been considered as a malignancy resistant to radiotherapy. There are no randomised studies on the efficacy of radiotherapy for melanoma although some clinical studies have shown benefit acquired from radiotherapy (Ballo and Ang 2003). For primary melanoma, radiotherapy is rarely indicated as an initial treatment option. It can be considered in special cases if surgery is not possible because of either the localisation or large size of the tumour or because of patient-related reasons (Ballo and Ang 2003). Postoperative radiotherapy can be delivered to lower the risk of local recurrence if the resection margins of the primary tumour are inadequate and if re-excision is not possible (Ballo and Ang 2004). Consensus has not been reached about the radiotherapy indications for localised disseminated melanoma, stage III (Ballo and Ang 2004). According to some studies, radiotherapy can be considered as adjuvant treatment after complete lymph node dissection to reduce the risk of nodal basin recurrence in patients with clinically involved nodes, numerous metastatic lymph nodes, or any metastatic node larger than 3 cm, metastatic involvement of the cervical or parotid region, or tumour growth outside the lymph nodes (Lee et al. 2000, Shen et al. 2000). For stage IV melanoma, radiotherapy can be used to palliate the neurological disorders, pain or compression symptoms caused by distant metastases. Stereotactic radiotherapy provides an alternative therapeutic strategy for single brain metastases (Atallah and Flaherty 2005).

1.8.3. Adjuvant therapy

To reduce the high risk (50%) of recurrences, postsurgical adjuvant therapy is considered for high-risk primary melanoma patients with thick (› 4 mm) tumours and/or locoregional metastatic lymph nodes (Balch et al. 2001a). An optional strategy in practice is treatment with interferon-α (INF-α). Several randomised trials have compared INF-α with control as adjuvant therapy for high-risk melanoma patients. The results of the individual trials on survival benefit have, however, been somewhat inconclusive or even conflicting, possibly because of the difference in dosages (high/intermediate/low) and treatment durations of the INF-α used (Eggermont 2001, Middleton and Thatcher 2001, Kirkwood et

al. 2002, Kefford 2003). Randomised trials have shown that high-dose INF-α is the only treatment showing activity against melanoma, improving 5-year relapse-free survival (RFS) by approximately 10% compared with surgery alone. The impact of high-dose IFN-α on overall survival (OS) has been less clear (Kirkwood et al. 1996, Kirkwood et al. 2000). Meta-analysis comprising twelve trials consisting of comparisons of INF-α with control demonstrates some evidence for dose-response in terms of RFS but not of OS; hence, uncertainty remains as to the benefits of IFN-α in melanoma (Wheatley et al. 2003). The use of INF-α as adjuvant therapy for primary melanoma patients has therefore to be evaluated in casu (Schuchter 2004).

Melanoma vaccines are designed to elicit a host immune response to tumour-associated antigens. Autologous and allogeneic whole-cell preparations as well as purified gangliosides, peptides, shed antigens and mechanical or viral melanoma cell lysates have been tested in laboratory or clinical studies (Livingston 2001). Thus far, randomised trials have not shown any significant benefit for survival in adjuvant therapy for primary melanoma. The results of several ongoing trials are, however, awaited (Sosman et al. 2004). For the time being, the vaccine therapy is only indicated for melanoma patients participating in clinical trials.

2. Biology of melanoma

The melanoma cell is the malignant counterpart of the normal melanocyte. Melanocytes located in the basal layer of the epidermis synthesise melanin pigment, which protects the body by absorbing solar ultraviolet light. The presence of melanocytes has also been confirmed in mucosa in the head and neck, oesophagus, small bowel, anorectum and urogenital tract. Primary mucosal melanoma represents a rare malignancy that is often diagnosed at a later stage and has a poorer prognosis than cutaneous melanoma (Chang et al. 1998). There are three types of ocular melanoma: uveal, cutaneous and conjunctival, all of which differ in epidemiology, pathogenesis and metastatic pattern (Devron and Char 2003). In an American cancer database report of 84 836 melanoma patients, 91.2% had cutaneous, 5.2% ocular, 1.3% mucosal and 2.2% unknown primary tumours (Chang et al. 1998).

Melanoma develops as a result of accumulated abnormalities in genetic pathways within the melanocyte. These abnormalities promote cell proliferation and increase apoptosis resistance (Meyskens et al. 2004). Genetic changes are expected to take place at the transition from a benign nevus to a dysplastic nevus, in situ melanoma or radial growth phase melanoma. Additional genetic activation is thought to take place at the transition from the radial to the vertical growth phase of melanoma, which has an invasive and expanding growth pattern (Herlyn and Satyamoorthy 2001). Melanoma cells of the vertical growth phase have escaped from the control of keratinocytes and migrated into the dermis, where they proliferate independently of exogenous growth factors, tumour cell growth relying more on autocrine stimulation and the influence of the tissue microenvironment (Sections 2.4. to 2.7.) Vertical growth phase melanoma cells are tumorigenic and have metastatic competence (Herlyn and Shih 1994). This has been shown both in human patients and in experimental animal models (Kath et al. 1991, Juhasz et al. 1993). In the course of melanoma progression, tumour cells invade the surrounding host tissue stroma, separate from the primary tumour mass and migrate through the extracellular matrix to enter into the lymphatic or vascular system. In close contact with neighbouring cells, cell adhesion molecules direct and organise the interaction between cells (Johnson 1999). Angiogenesis, the formation of new vasculature, is needed for the tumour to attain a diameter exceeding 1 – 2 mm (Folkman 1990).

2.1. Cell cycle regulation

Tumour growth is regulated by a balance between cell proliferation, growth arrest and cell death. The normal cell cycle is regulated by multiple proteins interacting in a fine, complex sequence. Mitogenic growth factors promote the entry of quiescent cells into

the first gap phase (G1) and the initiation of DNA synthesis (S phase) of the cell cycle. The second gap phase (G2) is then followed by mitosis.

The retinoblastoma (Rb) tumour suppressor protein in the G1 phase constitutes a key pathway known to be dysregulated in many malignancies. In melanoma pathogenesis, the p16-Cyclin D/Cdk4-pRb pathway functional unit is frequently altered, the function of Rb commonly being inhibited by hyperphosphorylation. Loss of p16 function is associated with progression of disease (Straume et al. 2000).

Ki-67, proliferating cell nuclear antigen, PCNA (a polymerase accessory protein, peaking in the G1/S phase), S phase fraction and silver-stained nucleolar organiser regions (AgNORs) are all reliable and widely used means for measuring the growth fraction, *e.g*. the proliferation index, and have been shown to be independent prognostic factors in small melanoma cohorts (Stone et al. 1996, Karjalainen et al. 1998, Barzilai et al. 1998, Korabiowska et al. 2000). In addition, cell-cycle regulators such as cyclins A and D3 correlate with growth fraction markers and independently predict prognosis in subsets of patients with melanoma (Florenes et al. 2000, Florenes et al. 2001). Accordingly, several studies have associated mitotic count with decreased survival of melanoma patients (Retsas et al. 2002, Azzola et al. 2003).

c-myc is a gene acting as one of the central regulators of cell proliferation. It can induce G1 entry in the absence of external growth factors. Moreover, *c-myc* can initiate apoptotic cell death in the presence of wild-type p53 protein or under conditions of growth factor deprivation. In subgroups of patients, *e*.g. those with acral primary melanomas, *c-myc* gene amplification has been an independent adverse prognostic factor (Ross and Wilson 1998, Grover et al.1999).

2.1.1. Ki-67

Ki-67 is a high molecular weight nuclear protein that is expressed during the cell cycle in late G1, M, G2 and S phases but not during early G1 and G0 phases or resting cells; thus it is a marker of proliferation. Mib-1, a monoclonal antibody used to detect Ki-67 in paraffin-embedded tissue, was first described by Gerdes (Gerdes et al. 1984).

In primary melanoma of the skin, proliferative activity has been studied by several methods, *e.g.* mitotic counts, flow cytometry and immunohistochemical staining with Ki-67 antibody. Mutation of cell cycle regulators can lead to an increased percentage of cells entering the S phase, thus causing proliferation of a tumour. Some authors have described an increase in proliferative activity in primary melanoma with increasing tumour thickness (Moretti et al. 1990, Rieger et al. 1993, Hazan et al. 2002). The use of proliferative markers, such as Ki-67, as indicators of clinical outcome has been demonstrated in several types of malignant tumour, *e.g*. breast cancer (van Dienst et al. 2004), lung cancer (Martin et al. 2004), and renal cell carcinoma (Kallio et al. 2004). Some studies have reported associations between increased Ki-67 expression and poor survival in thick melanomas (Ramsay et al. 1995a, Böni et al. 1996, Vogt et al. 1997). However, studies on tumours with a wider range of thickness have not

found any correlation between Ki-67 expression and disease outcome (Reddy et al. 1995, Talve et al. 1996a, Stone et al. 1996, Hazan et al. 2002). One study has demonstrated differing correlations between Ki-67 expression and prognosis, depending on tumour thickness; positive Ki-67 immunoreactivity was associated with a higher risk of metastasis in melanomas equal to or less than 1.5 mm thick and with a lower risk of metastasis in melanomas thicker than 1.5 mm (Moretti et al. 2001).

2.2. p53

The *p53* gene was discovered in the late 1970s encoding a cellular 53kd nuclear phosphoprotein called the wild-type protein p53. *p53* has characteristics of a tumour-suppressor gene as a controller of the G2/M checkpoint of the cell cycle, a participator in DNA repair and an inductor of G1 arrest of cell proliferation (Hussein et al. 2003). It inhibits DNA synthesis following DNA damage. *p53* also plays a role in programmed cell death by decreasing expression of Bcl-2 and increasing that of Bax (Harris and Hollstein 1993, Miyashita et al. 1994). Additional functional properties of p53 are its ability to activate many promoters and interact with numerous cellular and viral proteins. Loss of these functions, either through mutations or by allelic deletion, leads to enhanced genomic instability, gene amplification and changes in DNA ploidy (Livingstone et al. 1992). These alterations are associated with transformation in vitro and development of neoplasias in vivo (Levine et al. 1991) with resistance of p53-dependent apoptosis.

p53 is a frequently mutated gene, with more than 50% of human malignancies harbouring defects in it (Levine et al. 1991, Lee et al. 1994). UVR has been documented to cause *p53* mutations in squamous cell carcinoma and melanoma of the skin (Weiss et al. 1993, Ziegler et al. 1994). *p53* mutations have been detected in up to 60% of squamous cell carcinomas, in contrast to melanomas where mutations are rare, less than 5% (Ziegler et al. 1994, Hussein et al. 2003).

The mutant p53 protein has a half-life of several hours, in contrast to the wild-type protein´s half life of about 25 minutes (Reich and Levine 1984). Therefore the mutant form accumulates in the cell and is more easily detectable in immunohistochemistry. The p53 function can also be lost without mutations, as wild-type p53 can form inactive complexes with other proteins. High levels of wild-type p53 can be detected in benign cells of the skin with DNA damage caused by, for instance, UVR (Hall et al. 1996). Thus, a high level of p53 expression detected in immunohistochemistry is not necessarily caused by a mutated *p53* gene. This finding may partly account for the heterogeneous results of p53 expression in melanoma. In addition, the diversity of results obtained in studies on p53 alterations in melanoma may be attributed to differently assessed immunohistochemistry, the genetic heterogeneity of melanomas and the presence of several mechanisms for *p53* gene mutations (Hussein et al. 2003).

The clinical significance of *p53* mutations has been the subject of a number of controversies. Loss of p53 function has been correlated with shortened survival of patients with various carcinomas, *e.g*. of the breast, lung, colorectum and gastrum, in which it has been an unfavourable prognostic factor (Hollstein et al. 1997). Some studies on primary melanoma have shown no correlation between p53 expression and melanoma prognosis (Reddy et al. 1995, Weiss et al. 1995, Stone et al.1996, Talve et al. 1996b). Others, in contrast, indicate an association between high p53 expression and poorer disease outcome (Vogt et al. 1997).

2.3. Apoptosis

Apoptosis, *i.e.* programmed cell death, is induced by excessive exposure to UVR or DNA damage. *p53* is a central sensor responding to various stimuli to induce the apoptotic pathway although other apoptotic pathways also exist. In melanoma, *p53* mutations can alter the apoptotic pathway, although the frequency of mutations is rare. Loss of p53 function leads to the survival of damaged cells, thereby allowing tumour development (Ziegler et al. 1994). In the p53 apoptotic pathway, cytochrome c is released from mitochondria, inhibited by anti-apoptotic proteins such as Bcl-2/Bcl-xL. In the apoptotic sequence, Apaf-1 is activated and complexes are formed with caspase-9 and the effector caspase family, leading to cell death. Apaf-1-mediated apoptosis is inhibited by Akt/PKB and by IAP (inhibitors of apoptosis proteins). It has been reported that melanoma cells can avoid apoptosis by inactivating Apaf-1 (Soengas et al. 2001). Survivin and livin, two members of the IAP family, can also regulate apoptosis. Strong survivin expression has been described in invasive and metastatic melanoma (Grossman et al. 1999).

2.3.1. Bcl-2

Bcl-2 is the acronym for the B-cell lymphoma/leukaemia-2 gene. *Bcl-2* belongs to a multigene family that consists of genes encoding proteins with different functions, as inhibitors of apoptosis (Bcl-2, Mcl-1 and Bcl-X_L) and as promoters of cell death (Bax and Bcl-X_S). Interactions among proteins in this family regulate the sensitivity of cells to apoptotic stimuli (Reed 1994). The *bcl-2* gene was first discovered through its involvement in B-cell malignancies, in which (14:18) chromosomal translocations activate the gene in the majority of follicular non-Hodgkin´s B-cell lymphomas (Tsujimoto et al. 1985). The consequence of this translocation is deregulation of *bcl-2* and overexpression of the protein product that resides predominantly in inner mitochondrial protein, though it is also found in the nuclear and cellular membranes (Ramsay et al. 1995b). The detailed mechanism of action at the biochemical level is still not known (Coultas and Strasser 2003). It has been proposed that altered *bcl-2* results in dysregulated programmed cell death, allowing cells that were programmed to die to persist and accumulate (Reed 1994). Accumulation of cells with aberrant *bcl-2* has had tumorigenic potential in

lymphoproliferative disorders in mice (McDonnell et al. 1989). The same translocation has been discovered in normal tissue and in other neoplasias. The prognostic role of Bcl-2 in clinical studies on different malignancies has varied: expression of Bcl-2 has correlated with prolonged patient survival, *e.g.* in non-small cell lung cancer (Kren et al. 2004) and in renal cell carcinoma (Kallio et al. 2004). However, in other forms of cancer, *e.g.* prostatic cancer (Quinn et al. 2005) and classical Hodgkin´s lymphoma (Sup et al. 2005), expression of Bcl-2 has been an adverse predictor of the disease.

Human primary melanoma expresses Bcl-2 in up to 90% of cases (Cerroni et al. 1995, Tron et al.1995, Grover and Wilson 1996). In normal skin, Bcl-2 is detected as cytoplasmic staining along the basal layer of the epidermis, with variation depending on the localisation of the samples (van den Oord et al. 1994). Bcl-2 is also expressed in normal melanocytes (Ramsay et al. 1995b). The importance of Bcl-2 in the development and progression of melanoma is still disputed. Some studies report that its expression in primary melanoma is lower than in benign nevi (Ramsay et al. 1995b, Tang et al. 1998), whereas another study demonstrates an increase in Bcl-2 during disease progression (Leiter et al. 2000). Yet other investigators showed that Bcl-2 levels did not change during tumour progression and did not differ from expression in normal melanocytes (Cerroni et al. 1995, Plettenberg et al. 1995, Selzer et al. 1998). In addition, some studies have demonstrated an inverse correlation between increasing tumour progression to metastasis and Bcl-2 expression (van den Oord et al. 1994, Ramsay et al. 1995b, Tron et al. 1995). An association was reported between poor outcome and maintenance of Bcl-2 expression in regional metastatic lesions (Grover and Wilson 1996). In one study analysing changes in Bcl-2 expression during the course of the disease from primary melanoma to metastatic phase, Bcl-2 expression was higher in primary melanomas that later metastasised than in controls in which the disease remained localised, a finding suggesting a possible predictive role for Bcl-2 expression (Hernberg et al. 1998).

2.4. Growth factors

Melanoma cells express different growth factors and cytokines and their receptors during tumour progression, which, through autocrine and paracrine effects, enable the cells to grow autonomously and confer competence to metastasis (Lazar-Molnar et al. 2000). Autocrine growth factors, *e.g.* basic fibroblast growth factor (bFGF), interleukin-8 (IL-8), IL-10, melanoma growth stimulatory activity/growth-regulated protein (MGSA/GRO) and platelet-derived growth factor A (PDGF-A), produced by melanoma cells stimulate proliferation of the producing cell itself, whereas paracrine growth factors, *e.g.* platelet-derived growth factor (PDGF), endothelial growth factor (EGF), transforming growth factor-β (TGF-β), IL-1, insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF), contribute to the tumour growth indirectly, affecting the microen-

vironment of the tumour or suppressing the immune system, thus making it beneficial for tumour growth, invasion and metastasis (Halaban 1996). Host tissue cells for their part produce various activating growth factors, *e.g.* insulin growth factor-I (IGF-I), PDGF and scatter factor, as well as inhibitory growth factors, *e.g.* IL-1, IL-6, TGF-β, tumour necrosis factor (TNF) and interferons for melanoma cells. Inhibitory effects occur mainly in early-stage melanomas, diminishing and disappearing as the tumour progresses. In some cases, an inhibitory growth factor can switch to an autocrine stimulator when the tumour itself begins to produce it in an autocrine manner, thus enhancing its own proliferation (IL-6, TGF-β) (Lazar-Molnar et al. 2000).

Clinical studies have correlated IL-8 expression with the patient´s clinical course, with time to progression being significantly reduced in primary tumours expressing IL-8 in either the tumour cells or keratinocytes of the overlying epidermis (Nurnberg et al. 1999). In another study, IL-8 over-expression was discovered in primary melanomas compared with metastatic samples of the same patients, however, no correlation was found between IL-8 expression and survival (Gutman et al. 2002).

The secretion of the lymph vessel-specific VEGF-C and -D of melanomas has been associated with the formation of new lymph vessels and an increased risk of lymph node metastases (Nathanson 2003).

2.5. Angiogenesis

Angiogenesis refers to the formation of new blood vessels. Induction of angiogenesis is required for most tumours to grow beyond 1 to 2 mm in diameter, the limit of the simple diffusion of nutrients and oxygen (Folkman 1990). Angiogenesis is controlled by angiogenic factors that can be secreted by tumour cells, inflammatory cells and stromal cells (Fox 1997). Several angiogenic growth factors have been identified, including VEGF, bFGF, PDGF, TGF-α and -β and angiogenin, as well as angiogenic inhibitors *e.g.* angiostatin, thrombospondin-1, endothelial monocyte activating polypeptide II and endostatin (Srivastava et al. 2003). In contrast to nonneoplastic angiogenesis, which has a certain end-point, a tumour induces angiogenesis almost continuously until the neoplasm is eliminated or the host dies (Folkman and Klagsbrun 1987). Moreover, tumour-associated endothelial cells are stimulated to degrade the basement membrane and to migrate into the perivascular stroma. Migrating endothelial cells produce type IV collagenase and other members of the matrix metalloproteinase family (Ausprunk and Folkman 1977). Proliferating endothelial cells also release growth factors that may stimulate the tumour (Folkman 1992).

Angiogenesis permits solid tumour growth. For clinical metastases to occur, tumour cells must first enter the circulation, evade the host immune response, adhere to the capillary wall in a suitable tissue, leave the circulation and then establish a new tumour vasculature that will permit growth. Tumour vascularity has been proposed as

a prognostic marker for a number of solid tumours such as lung cancer (Singhal et al. 2005), prostatic cancer (Quinn et al. 2005), head and neck squamous cell carcinoma (Smith et al. 2001) and breast cancer (Choi et al. 2005). These studies indicate that an increased number of microvessels within the primary tumours correlates with a higher metastasis rate or recurrence. The capacity of cutaneous melanoma to induce angiogenesis is well established (Fallowfield and Cook 1991, Barnhill et al. 1992), but the importance of angiogenesis in melanoma progression is controversial.

It has been demonstrated that in situ tumours are avascular and that the onset of angiogenesis ushers in a phase of rapid growth (Folkman and Klagsbrun 1975). In contrast, other studies have shown that there has been increased vascularity in the dermis beneath melanoma in situ, suggesting that tumour angiogenesis precedes invasion and that invasion and metastatic spread are due to some other intrinsic change in the melanoma cells themselves (Fallowfield and Cook 1991). The prognostic role of tumour vascularisation in melanoma is undetermined, some studies reporting that increased vascularity in melanoma is associated with a poorer prognosis (Srivastava et al. 1988, Rongioletti et al. 1996, Neitzel et al. 1999, Kashani-Sabet et al. 2001, Zamolo et al. 2001), others finding no such association (Carnochan et al. 1991, Barnhill et al. 1994, Busam et al. 1995, Lin et al. 1999).

2.6. Adhesion molecules

Adhesion molecules can be grouped into integrins, the immunoglobulin superfamily and cadherins. Integrins link the cell membrane to the cytoskeleton, mediate adhesion to extracellular matrix (ECM) components and to other cells, and transfer signals to the cell inducing changes in gene expression (van der Flier and Sonnenberg 2001). Integrins α4β1 and αvβ3 have been correlated with invasive and metastatic potential in melanoma cell lines. Expression of αvβ3 integrin has predicted a short recurrence-free interval in clinical stage I and short overall survival in intermediate thickness melanoma (Natali et al. 1997, Hieken et al. 1999).

As well as mediating adhesion, members of the immunoglobulin superfamily serve as receptors for integrins and ECM proteins. One of these, melanoma cell adhesion molecule (MCAM; previously known as Mel-CAM or MUC18), is a cell surface glycoprotein that is rarely observed in nevus cells but is increased in melanoma cell lines, correlating directly with enhanced growth and metastatic capacity of the cells (Luca et al. 1993, Xie et al. 1997). MCAM is also upregulated as melanomas enter the vertical growth phase (Newton-Bishop 1997), but to date there are no reports of the prognostic impact of MCAM in melanoma. Expression of intercellular adhesion molecule-1, ICAM-1, is strongly upregulated in melanoma cells as compared with benign melanocytic lesions. Increased expression is associated with poor prognosis in stage I melanomas (Natali et al. 1997). Activated leukocyte cell adhesion molecule

(ALCAM) is thought to mediate clustering of melanoma cells, and its expression has been correlated with increasing vertical thickness (van Kempen et al. 2000). No prognostic studies exist on ALCAM in melanoma. Cell surface glycoprotein CD44 and its ligand, extracellular matrix component hyaluronan (HA), maintain adhesive restraints between the cells in normal epidermis (Soukka et al. 1997). Although some studies suggest that CD44 and HA enhance the growth and metastatic capacity of melanoma cells (Guo et al. 1994, van Muijen et al. 1995), the clinical importance of CD44 expression for prognosis remains controversial (Harwood et al. 1996, Dietrich et al. 1997).

Cadherins, both E- and N-subtypes, are important molecules in the morphogenesis and maintenance of skin structure, and they determine the location of melanocytes in the skin (Tang et al. 1994). In normal skin, E-cadherin is expressed on the cell surface of keratinocytes and melanocytes whereas N-cadherin is expressed by fibroblasts and endothelial cells. In vitro and in vivo studies report that during melanoma progression, there is a switch from the predominance of E-cadherin to that of N-cadherin, freeing melanocytes from the control of keratinocytes and enabling them to interact directly with other N-cadherin-expressing cells such as vascular endothelial cells (Hsu et al. 1996, Seline et al. 1996). One clinical report on melanomas with high expression of E-cadherin together with S100A4 negativity noted a significant association with favourable disease-free survival (Andersen et al. 2004).

2.7. Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are proteolytic enzymes that remodel and degrade the tumour surrounding extracellular matrix and basement membrane in the process of tumour invasion. MMPs are secreted from melanoma cells and from surrounding cells of the stroma. Increased expression of MMP-1, -2, -9 and -13 has been correlated with an invasive melanoma phenotype in human melanoma cell lines (Hofmann et al. 2000). High MMP-2 immunoreactivity in melanoma cells of the primary tumour has been reported to be an independent unfavourable prognostic factor and correlated with later haematogenous metastasis (Väisänen et al. 1996, Väisänen et al. 1998). In a clinical series of primary melanoma there was no clear association with the immunohistochemical expression of MMP-2 and tumour progression, and serum levels of MMP-2 and MMP-9 were not specific in showing melanoma progression (Redondo et al. 2005). Previous reports have suggested that high expression levels of MMP-1 and MMP-3 have correlated with shorter disease-free survival in human metastatic melanoma (Nikkola et al. 2002) and that patients with high serum MMP-9 levels have had significantly poorer OS than patients with lower serum MMP-9 levels (Nikkola et al. 2005).

2.8. Tenascin-C

Tenascin-C (Tn-C) is a member of the extracellular matrix (ECM) protein family. ECM is composed of various molecules such as hyalyronic acid, proteoglycanes, glycosaminoglycanes, elastin and collagen, and of glycoproteins such as fibronectin, vitronectin, laminin and tenascin. ECM forms a supporting network to which cells adhere; further it acts as an information system in coordinating signals originating from the tissue microenvironment and adjacent cells (Chiquet-Ehrismann and Chiquet 2003).

The human tenascin group includes tenascin-C, -R, -X and -W. Discovered independently by several research groups, Tn-C is the founding member of the family, as a protein enriched in the stroma of the gliomas (Bourdon et al. 1983) and as a myotendinous antigen (Chiquet and Fambrough 1984). Tn-C is a large glycoprotein consisting of six identical subunits built from variable numbers of repeated domains, each domain including heptad repeats, endothelial growth factor (EGF)-like repeats, fibronectin type III domains and a C-terminal globular domain (Jones and Jones 2000). In cell cultures, several Tn-C isoforms have been identified (Latijnhouwers et al. 2000). Tn-C is expressed transiently during embryogenesis and foetal development and in a restricted pattern at sites of cell proliferation, migration and ECM remodelling in adult tissues. Correspondingly, in clinical settings, Tn-C is expressed in acute inflammation, wound healing, regeneration and neoplastic processes in adult tissues (Jones and Jones 2000).

Tn-C is synthesised mainly by dermal fibroblasts but, in some conditions, by epidermal keratinocytes, too (Latijnhouwers et al. 2000). *In-situ* hybridisation studies have shown that tumour cells can also act as a source of Tn-C, as in melanomas and in basal cell and squamous cell carcinomas (Herlyn et al. 1991, Tuominen et al. 1997). Tn-C is not detected in normal mesencymal tissues, *e.g.* muscle or adipose or fibrous connective tissue, but neo-expression of Tn-C has been shown in more than 50% of the rhabdomyosarcomas, fibromas and liposarcomas (Schnyder et al. 1997). Expression of tenascins is regulated by cytokines, vasoactive peptides, other ECM proteins, biomechanical factors, and a variety of growth factors, *i.e.* members of the TGF-β family (Jones and Jones 2000, Chiquet-Ehrismann and Chiquet 2003).

The function of Tn-C is complex, and Tn-C isoforms have been found to have different effects on the adhesion, migration and proliferation of cultured cells (Latijnhouwers et al. 2000). In experimental set-ups, Tn-C can act adhesively for one cell type and inhibit the adhesion of another. The effect of Tn-C on a single cell may also vary, depending on the cell´s contacts with the other ECM components. Previous studies show that tenascins generally interfere with the integrin-dependent spreading of most cell types (Chiquet-Ehrismann and Chiquet 2003).

The suggested role of Tn-C in malignant neoplasias is complex. As Tn-C contains epidermal growth factor-like repeats, it may induce tumour growth through an autocrine mechanism (Engel 1989, Jones and Jones 2000). Experimental studies show

that it also seems to be involved in angiogenesis. Tn-C stimulates endothelial-cell migration and mitogenesis and growth-factor-dependent endothelial cell sprouting. In addition, Tn-C is a major constituent of the ECM surrounding angiogenic blood vessels (Jones 2001). ECM proteins take part in stromal-epithelial interactions during tumour invasion. The basement membrane is lost during cell invasion and Tn-C may be involved in the detachment of cancer cells, promoting their invasive potential (Shintani et al. 1997).

In the normal human skin Tn-C is expressed in the upper dermis, where it may be virtually absent or reveal patchy expression along the basement membrane. Immunohistochemical staining is intense around hair buds, eccrine glands and the intima of larger blood vessels. Similar strong expression of Tn-C has been detected along the basal membrane, extending to the deeper dermis in psoriasis and basal cell carcinoma, thus indicating its unspecific appearance in different tissues (Schalkwijk et al. 1991). Although Tn-C is not indicative of malignancy, its expression in melanocytic tumours is higher during tumour progression.

Moderate expression has been detected in benign and dysplastic nevi at the dermoepidermal junction and in the papillary dermis. In invasive melanomas, Tn-C expression has been stronger and has extended deeper into the reticular dermis. In melanoma metastases, Tn-C expression has been greatly increased. In addition, intracytoplasmic Tn-C has been detected both in primary and in metastatic melanomas (Tuominen and Kallioinen 1994). To our knowledge, there are as yet no studies correlating the findings of Tn-C expression in melanoma and the clinical course of the disease.

Previous studies show that Tn-C expression increases during disease progression from benign to preinvasive and to invasive forms, *e.g.* in colorectal tumours (Riedl et al. 1992), endometrial tumours (Sasano et al. 1993), transitional cell tumours of the urinary bladder (Tiitta et al. 1993), gastric tumours (Ilunga and Iriyama 1995), adenoid cystic tumours of the salivary glands (Shintani et al. 1997), lung tumours (Kusagawa et al. 1998), prostate tumours (Xue et al. 1998), laryngeal tumours (Yoshida et al. 1999) and breast tumours (Goepel et al. 2000).

An association with increased Tn-C expression in malignant tumours and enhanced malignant behaviour of the disease has been established in several studies, Table 4.

Table 4
Studies on the positive association between increased Tn-C expression and enhanced malignant behaviour of the disease.

Author	Tumour
Harada et al. 1994	oral squamous cell carcinoma
Jahkola et al. 1996	breast cancer
Jahkola et al. 1998	breast cancer
Tanaka et al. 2000	osteosarcoma
Aishima et al. 2003	intrahepatic cholangiocarcinoma
Leins et al. 2003	brain tumours
Koljonen et al. 2005	Merkel cell carcinoma

A study on 52 astrocytic brain tumours reported a correlation between Tn-C expression in tumour vessels and the tumour network and the degree of histological malignancy. It was suggested that Tn-C in tumour vessels was produced either by the tumour cells or by the vascular components with the induction of the surrounding tumour cells. These findings associated Tn-C with neoplastic angiogenesis (Kim et al. 2000).

Contradictory findings report that in malignant salivary gland tumours (Karja et al. 1995), the majority of the tumours were Tn-C-negative and that Tn-C immunoreactivity was not associated with the clinical behaviour of these tumours. In clinical reports of invasive breast cancer and colon cancer, patients with Tn-C positive tumours have had a better prognosis than those with Tn-C negative tumours (Shoji et al.1993, Iskaros et al. 1997).

In clinical practice, Tn-C has been used in therapeutic studies of malignant gliomas. In a phase I trial, newly diagnosed patients with malignant gliomas but with no prior radiation therapy or chemotherapy were treated with a single injection of ^{131}I radiolabelled anti-Tn monoclonal antibody administered clinically into surgically created resection cavities. The results have been encouraging in respect of the survival of the patients, the toxicity of the therapy and the prevalence (only 2.5%) of debulking surgery for symptomatic radiation necrosis (Cokgor et al. 2000).

2.9. Ezrin

Ezrin, radixin and moesin constitute the ERM (ezrin-radixin-moesin) intracellular protein family (Berryman et al. 1993, Tsukita et al. 1997, Vaheri et al. 1997), which is known to mediate an interaction between actin microfilaments and cell membranes. The proteins are thought to play a key role in the control of cell morphology (Vaheri et al. 1997, Hiscox and Jiang 1999). Members of the ERM family appear to have three structural domains: N-domain, α-domain and C-domain with a high percentage (72–75%) of amino-acid sequence homology. Merlin (or schwannomin), a tumour suppressor protein defective or absent in schwannomas and meningeomas and mutated in neurofibromatosis 2, is a diverged member of the ERM family, having only 49% identical protein structure (Louvet-Vallee 2000). The functional properties of merlin are thus considered to differ somewhat from those of ERM proteins. The ERM proteins each exhibit both a tissue-specific expression pattern in the whole body and a restricted pattern of expression. Ezrin is expressed in a wide variety of epithelial and mesothelial cells, *e.g.* at high levels in the small intestine, stomach, lung, pancreas and kidney (Gould et al. 1989, Berryman et al. 1993). The ERM proteins are concentrated in actin-rich surface structures just beneath the plasma membrane; ezrin is specifically localised in microvilli and in membrane ruffles (Berryman et al. 1993).

Ezrin, the best studied member of the ERM family, is a 70kDa phosphoprotein purified in 1983 (Bretscher 1983). Like the other ERM proteins, ezrin exists in two forms: an active state that is localised beneath the plasma membrane, and a dormant state that is soluble in the cytoplasm. The tyrosine phosphorylation of a C-terminal residue of ezrin is mediated by the Rho pathway and induced by several growth factors, *e.g*. EGF and HGF/Scatter factor (Vaheri et al. 1997, Hiscox and Jiang 1999). Phosphorylation of ezrin induces transition of the protein from an inactive to an active form (Krieg and Hunter 1992).

Ezrin binds actin filaments by their C-terminal domains, and cell surface adhesion molecules such as CD43 and CD44, ICAM-1, -2, -3 and syndecan 2 by their globular N-terminal domains (Kaul et al. 1996, Heiska et al. 1998). As well as acting as a structural linker between the plasma membrane and the actin cytoskeleton, ezrin is believed to relay signals between the surface and the interior of the cell. Ezrin is also suggested to be involved in cell-cell and cell-substrate adhesion events (Hiscox and Jiang 1999) and in microvilli formation (Takeuchi et al. 1994, Kaul et al. 1996). Microvilli furthermore are considered to play an important role in the early step of tumour invasion (Weiss 1976). Microvilli have been demonstrated to disappear from the cell surface of thymoma cells when cultured in the presence of antisense oligonucleotides, which suppress expression of ERM proteins. This leads to the destruction of cell-cell and cell-substrate adhesion (Takeuchi et al. 1994), as was noted in colorectal cancer cell lines, too (Hiscox and Jiang 1999). These observations provide further evidence of the role of ezrin in cell motility (Hiscox and Jiang 1999).

The ERM proteins merlin and moesin have been demonstrated to act as tumour suppressors. A deficiency of merlin encoding neurofibromatosis 2 was associated with the development of a variety of cancers and a high rate of metastasis (McClatchey 2003). The immunoreactivity of moesin was diminished in metastatic lesions in the skin and lymph node of melanoma (Ichikawa et al. 1998). In contrast to these findings, other studies indicate an active role for ezrin in promoting cell growth, invasiveness and metastasis. In vitro studies suggest that expression of ezrin in cell lines of endometrial (Ohtani et al. 1999), pancreatic (Akisawa et al. 1999) and colorectal carcinoma (Hiscox and Jiang 1999) plays a role tumour cell morphology, invasion and migration.

In a mouse rhabdomyosarcoma, ezrin expression correlated significantly with metastasis (Yu et al. 2004). The same was discovered in a mouse model of osteosarcoma, where suppression of ezrin reduced lung metastases in mice (Khanna et al. 2004). In a clinical study on dogs with osteosarcoma, the presence of strong ezrin expression was associated with shorter disease-free intervals than in dogs with low expression of ezrin in primary tumours (Khanna et al. 2004). Similarly, in a study on paediatric patients with osteosarcoma, strong ezrin expression in primary tumours was associated with shorter disease-free intervals than in patients showing low ezrin expression (Khanna et al. 2004). In a small group of paired primary and metastatic lesions of endometrial cancers, expression of ezrin was higher in metastatic than in matched primary samples (Ohtani et al. 2002). In astrocytic tumours of the brain, an increase in ezrin immunoreactivity correlated with increased malignancy of the tumours (Geiger et al. 2000). In a study of uveal melanoma, two-thirds of the specimens were immunoreactive for ezrin. The presence of ezrin immunoreactivity was associated with higher mortality and with two independent high-risk characteristics: microvascular density and number of macrophages in tumours (Mäkitie et al. 2001). In serous ovarian carcinomas, in contrast to other previous reports, negative or weak immunoreactivity correlated with poor patient outcome (Moilanen et al. 2003).

AIMS OF THE STUDY

The prognosis of primary melanoma is good if it is diagnosed at an early phase and treated radically. Once the tumour has metastasised, the prognosis deteriorates and no specific curative treatment is available. The established prognostic factors do not always identify the high-risk patients who should be followed up intensively and selected for adjuvant therapies. The main purpose of this study was to identify tumour-related markers for predicting the behaviour of primary melanoma and to evaluate the prognosis for melanoma patients in a clinical series of the Finnish population.

The specific aims of the study were:

1. To define the overall prognosis of melanoma in a series of patients treated and followed up in a single unit to evaluate patient- and tumour-related prognostic factors for survival (Study I)

2. To investigate the expression and prognostic significance of Ki-67, Bcl-2 and p53 in primary melanoma and to analyse the behaviour of these markers during disease progression (Study II)

3. To examine the role of tumour vascularisation as a prognostic factor in primary melanoma (Study III)

4. To evaluate the expression patterns and prognostic significance of tenascin-C in primary melanoma (Study IV)

5. To explore the expression of ezrin in primary melanoma and to assess its correlation with the course of the disease (Study V)

MATERIALS AND METHODS

1. Patients (Studies I-V)

The subjects in Study I consisted of all patients diagnosed with primary melanoma and registered as primary melanoma patients at the Department of Plastic Surgery, Helsinki University Hospital, Helsinki, Finland, between 1998 and 1991. Eight of the original 306 patients were excluded because re-examination of their tumours did not confirm the diagnosis, leaving 298 patients to form the entire patient population in Study I. Subgroups of patients for Studies II-V were selected from the patient population in Study I on the basis of the concurrent availability of tumour blocks, and consisted partly of the same patients. The disease history and the clinical data on patient characteristics and surgical treatment and on consultations and treatments by oncologists were gathered from the patients´ records.

The patients were followed up postoperatively at 3-month intervals for the first 2 years, and thereafter every 6 months for up to 5 years. Special attention focused on the surgically treated area and the lymph nodes and on any suspicious skin lesions elsewhere in the body. Radiological examinations and fine-needle biopsies were conducted on the basis of the clinical status. The final status was determined after a prospective clinical follow-up investigation and established whether the patient was disease-free, had developed recurrences or metastases or had died of melanoma. The data were collected from the patients´ records. The overall survival data indicating whether the patient had died of melanoma or of some other cause, or was still alive were obtained from the National Population Registry. The study was approved of by the Ethics Committee of the Department of Surgery, Helsinki University Hospital.

2. Tumours (Studies I-V)

The tissue samples were collected from various pathology laboratories, the majority of them located in Southern Finland. The immunohistochemical studies (II-V) were conducted on all tumours from the original material (Study I) that were currently available. The samples were re-examined by an expert pathologist to verify the melanoma diagnosis. The Breslow and Clark classifications were also re-assessed. The tumours were classified according to the 1992 AJCC staging system in classifying the tumours, Table 5. During the period in which the primary melanomas were re-examined, the presence of ulceration was not a major criterion for staging melanomas. This was not therefore systematically re-analysed and so the number of ulcerative tumours may have been un-

derestimated. At the time of our study the technique of detecting nodal micrometastases by examining the sentinel nodes was not yet in use. The data on patients and tumours in individual studies are presented in Table 6. The first metastatic tissue specimen of 12 patients was also analysed in Study V, and of 18 patients in Study II; these tissue samples were collected from the Department of Pathology, University of Helsinki, Helsinki, Finland. The metastatic samples were of local recurrent tumours of the skin (n = 4) and subcutis (n = 6) and of regional lymph node metastases (n = 8).

Table 5
Staging system for cutaneous melanoma according to AJCC in 1992

Criteria	TNM	AJCC
tumour thickness ≤ 0.75 mm and/or Clark level II[a]	pT1N0M0	IA
tumour thickness 0.76-1.5 mm and/or Clark level III	pT2N0M0	IB
tumour thickness 1.51-4 mm and/or Clark level IV	pT3N0M0	IIA
tumour thickness > 4 mm and/or Clark level V	pT4N0M0	IIB
Limited regional nodal metastases or fewer than five in-transit metastases	any pT,N1,M0	III
Advanced regional metastases or systemic metastases	any pT,N2,M0 or any pT, any N, M1 or M2	IV

[a]When tumour thickness and level of tumour invasion (Clark classification) do not coincide within the pT classification, thickness should be given precedence.

Table 6
Patient and tumour data (Studies I to V)

Parameter	Study I n = 298	Study II n = 117	Study III n = 84	Study IV n = 98	Study V n = 95
Prospective, clinical follow-up, median years (range)	4.8 (0.03-9.3)	4.6 (0.2-7.5)	4.05 (0.2-7.5)	3.9 (0.04-7.4)	4.8 (0.2-10.0)
Overall follow-up, median years (range)	9.5 (0.1-12.5)	10.0 (8.6-15.6)	4.05 (0.6-7.5)	9.3 (0.5-12.1)	9.1 (0.5-15.4)
Age at diagnosis, median years (range)	53.9 (17.0-88.2)	54.5 (24.0-88.0)	54.2 (20.8-88.2)	57.4 (24.0-84.6)	58.6 (24.0-84.6)
Male/Female	161/137	59/58	42/42	47/51	44/51
Tumour thickness, median mm (range)	1.2 (0.2-20.4)	1.2 (0.3-10.0)	1.2 (0.4-7.0)	1.0 (0.3-7.0)	1.2 (0.4-15.6)
Tumour invasion:					
Clark I	16	0	0	6	1
Clark II	36	7	3	8	7
Clark III	123	66	51	56	50
Clark IV	97	38	25	24	32
Clark V	11	5	4	3	4
Unclassified	15	1	0	0	1
Tumour ulceration:					
present	34	16	14	14	15
absent	264	101	70	84	80
Tumour location:					
Head or neck	38	12	10	12	11
Trunk	115	47	35	40	36
Upper extr. Lower	46	19	13	16	17
extr.	78	31	20	25	27
Palm	5	2	2	1	1
Sole of foot	10	5	3	4	3
Vulva	4	1	1	0	0
Perineum	2				
Recurrent disease:					
yes	73 (24%)	34 (29%)	17 (20%)	19 (19%)	26 (27%)
no	225 (76%)	83 (71%)	67 (80%)	79 (81%)	69 (73%)
Cause of death:					
Melanoma	53 (18%)	22 (19%)	9 (11%)	11 (11%)	16 (17%)
Other	46 (15%)	20 (17%)	1 (1%)	18 (18%)	20 (21%)
Alive	199 (67%)	75 (64%)	74 (88%)	69 (71%)	59 (62%)

3. Immunohistochemistry (Studies II–V)

Immunohistochemistry for detection of Ki-67, Bcl-2, p53 (n=117), CD-31 (n=84), and for tenascin-C (n=98) was performed at the Haartman Institute Laboratory, University of Helsinki and for ezrin (n=95) at the Laboratory of Pathology and Anatomy and Neuroscience Program, Biomedicum, University of Helsinki.

Sections 5µm thick were cut from paraffin-embedded tissue blocks, deparaffinised and rehydrated. The sections were pretreated for Ki-67, Bcl-2 and p53 with 10 mM sodium citrates buffer pH 6.0 in a microwave oven for 4x5 min and left in the buffer at room temperature for 20 min, and for ezrin with 10 mM sodium citrate buffer pH 6.0 at 95°C for 10 min. The sections were subjected to enzymatic digestion with Protease XXVIII (1mg/ml of phosphate buffered saline (PBS) at 37°C for 7 min) in the procedure for CD-31 and with pepsin (0.1% in 0.01 $_{M}$ HCl at 37°C for 30 min) for Tn-C. After antigen retrieval, the slides were incubated with primary antibodies. Binding of the primary antibodies was detected with specific secondary antibodies according to each manufacturer´s instructions. The sections were then counterstained with haematoxylin, Table 7.

Table 7
Immunohistochemistry

Antigen	Monoclonal Antibody	Dilution	Immunoperoxidase technique	Study
Ki-67	Mib-1 Immunotech. Marseilles France	1:500	VECTASTAIN® *Elite* ABC Kit, Vector Laboratories, Inc. Burlingame, CA, USA	II and V
Bcl-2	anti-Bcl-2 DAKO Glostrup Denmark	1:200	VECTASTAIN® *Elite* ABC Kit, Vector Laboratories, Inc. Burlingame, CA, USA	II and V
p53	anti-p53 DAKO Glostrup Denmark	1:300	VECTASTAIN® *Elite* ABC Kit, Vector Laboratories, Inc. Burlingame, CA, USA	II and V
CD-31	JC/70 DAKO Glostrup Denmark	1:50	DAKO StreptABComplex/ HRP Duet Mouse/ Rabbit Kit Glostrup Denmark	III
Tn-C	143DB7 Biohit Diagnostics Oy, Helsinki Finland	1:2000	alkaline phosphatase-coupled rabbit immunoglobulins to mouse IgG, Dakopatts Glostrup, Denmark	IV
ezrin	clone 3C12 Vaheri Finland	1:2000	ABC method, UltraVision, Lab Vision Inc., Fremont CA,USA	V

3.1. Quantification of immunohistochemistry (Studies II-V)

All immunohistochemical evaluations were performed blindly, without reference to the clinical data of the patients. The slides were scored independently by Ilmonen and Hernberg in Study II, Tarkkanen providing consultation assistance; by Ilmonen in Study III, Kariniemi providing consultation assistance; by Ilmonen, Turunen and Jahkola in Study IV; by Ilmonen and Carpen in Study V.

For Bcl-2, a cytoplasmic staining pattern was evaluated. The whole tumour area was examined, and the result was reported as the percentage of positively stained tumour area. In addition, the expression pattern of Bcl-2 was scored as absent, focal or diffuse. If focal, immunoreactivity was present only in scattered aggregates of cells; if diffuse, immunoreactivity extended throughout the tumour. Epidermal basal cells around the tumour in adjacent normal skin and small reactive peritumoral lymphocytes provided the positive control.

For Ki-67 and p53, a nuclear staining pattern was evaluated. In a tumour with heterogeneous Mib-1 or p53 immunoreaction, the spot in the tumour area that resembled the rest of the tumour was selected for analysis. The proportion of positive cells among 200 tumour cells was reported. Epidermal basal cells around the tumour in adjacent normal skin served as the positive control.

For the immunostaining of CD-31, the tumour area was defined with the AxioHOME morphometric system. Because tumours showed a heterogeneous distribution of vessels, an attempt was made to cover the whole tumour or most of the tumour area. The number of intratumoral vessels in a defined tumour area was then counted to obtain the blood vessel density. Blood vessel quantification was performed with light microscopy. The criteria for separate countable vessels were the presence of a vessel lumen and any red staining cells clearly separated from adjacent vessels, tumour cells and connective tissue elements. Cells staining red in the presence of a vessel lumen in the adjacent normal dermis served as the positive control, Figure 1.

For Tn-C, both extracellular expression in the stroma and cytoplasmic staining patterns were evaluated. The stromal expression of Tn-C for was scored either as negative or as focally or diffusely positive. The staining pattern was scored as focal if there were both Tn-C-positive areas and completely Tn-C-negative areas in the section of tumour examined, and as diffuse if the entire part examined was filled with tenascin. Beneath the basement membrane, a diffuse staining pattern was seen as a continuous band of Tn-C. In the stroma of the tumour, diffuse Tn-C appeared as a dense network around melanoma islets; and in the stroma of invasive regions diffuse Tn-C expression was seen as a continuous thin border. Cytoplasmic staining of Tn-C was either positive or negative. Epidermal basement membrane in adjacent normal skin served as the positive control, Figures 2a and b.

For ezrin, a cytoplasmic staining pattern was evaluated. Immunoreactivity was graded as negative, weak or strong. The colour intensity in the positive immunoreaction

determined whether the classification was weak or strong. The immunoreaction tended to be homogeneous throughout the tumour, with the few exceptions of tumours with varying intensity in different regions of the tumour. In these cases, a higher staining intensity in more than 20% of cells was classified in the higher category. Epithelial cells of the surrounding normal skin and the peritumoral lymphocytes served as the positive control, Figures 3a and b.

Negative controls for all studies were provided by omitting the primary antibodies and substituting irrelevant monoclonal antibodies.

4. Statistical analysis (Studies I-V)

Statistical analyses were performed with the Statview™ statistical program (Abacus Concepts Inc., Berkeley, CA, USA). Melanoma-related disease-free survival time was calculated as the time interval between diagnosis and the first recorded sign of disease progression after the primary operation. Melanoma-related overall survival was defined as the time interval between diagnosis and death from melanoma. Patients with no signs of progression at their last follow-up visit and patients who died of other causes were censored at the time of their last visit or death, respectively.

The distribution of parameters was characterised by standard descriptive methods. Contingency tables were analysed by chi-square test and Fisher´s exact test. The non-parametric Mann-Whitney U test, Kruskall-Wallis test and Spearman rank correlation test were used to analyse nonparametric variables. Multiple regression analysis was applied to show the diminishing of surgical margins during 1988-1991 in Study I. The Wilcoxon signed rank test was used to compare the values of Ki-67, Bcl-2 and p53 in primary and metastatic tumours in pairs. Univariate analysis of disease-free and disease-specific overall survival was based on the Kaplan-Meyer method, and differences in survival were tested with the Mantel-Cox (log-rank) test. Independent prognostic factors were identified with stepwise Cox multivariate analysis. p values < 0.05 were considered statistically significant in Studies I, II, III and IV, and ≤ 0.05 in Study V. Figure 8 presents data as a box plot in which each box is composed of five horizontal lines that display the 10^{th}, 25^{th}, 50^{th} (median), 75^{th} and 90^{th} percentiles of a variable such that the central 50% of the values falls within the range of the box. The central vertical lines in the boxes mark the medians. Values above the 90^{th} and below the 10^{th} percentile are plotted as points.

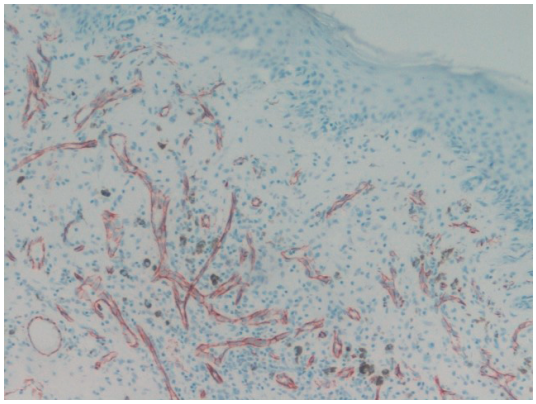


Figure 1
Rich vascularity associated with primary cutaneous melanoma visualised by endothelial immunohistochemical staining of CD-31.

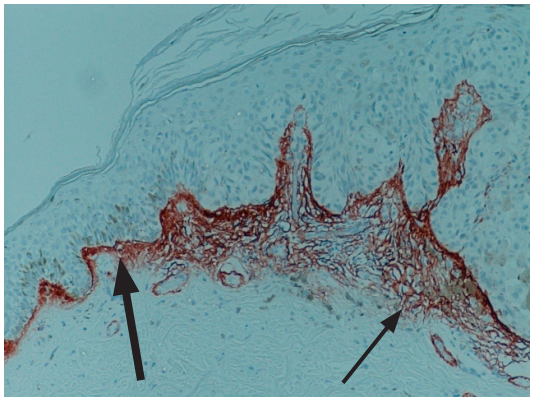
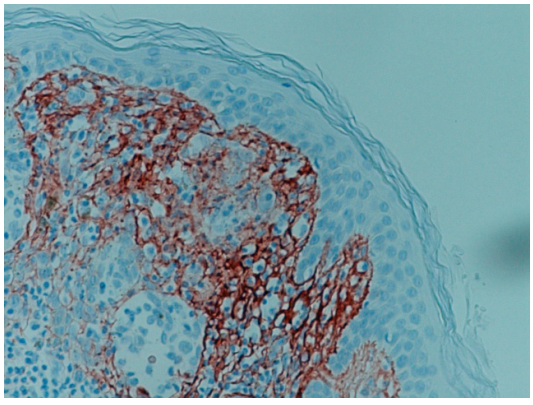


Figure 2
Tenascin-C immunoreactivity in primary melanoma specimens. Examples of tumours showing

a) diffuse Tn-C expression at the upper dermal lateral border (thick arrow) and at the deep, dermal border (thin arrow) in a superficial melanoma;



b) diffuse Tn-C expression in the tumour stroma forming a network around melanoma islets.

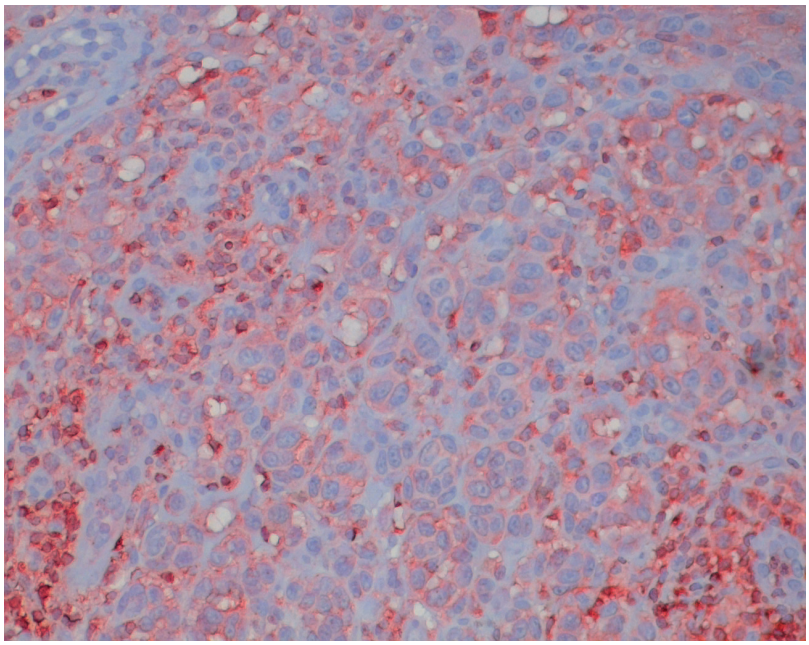
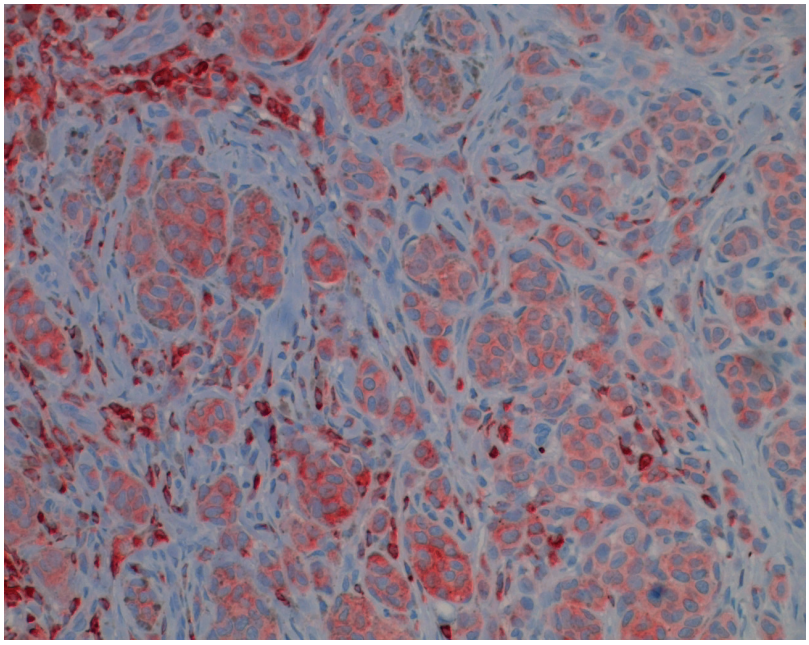


Figure 3
Ezrin immunoreactivity in primary melanoma specimens. Examples of tumours showing a) weak ezrin immunoreactivity in the stroma;



b) strong ezrin immunoreactivity in the stroma.
Note also strong immunoreactivity of tumour-associated lymphocytes in a) and b).

RESULTS

1. Study I

Most of the tumours (77%) had already been excised by general practitioners or surgeons before the patients were seen at their first visit to Helsinki University Hospital. The majority of the tumours were local, 91% being stage I or II. At the spot where a melanoma later developed, 42% of the patients had had a nevus for several years and 10% of them from early childhood or birth. However, 38% of patient records made no mention of a pre-existing nevus. The different histories of the nevus anamnesis seemed to have prognostic influence on survival. Melanomas arising straight from the normal skin were more often ulcerated (20.7%) and thicker (median tumour thickness 2.5 mm) and had a poorer prognosis than melanomas that had developed from a short-lived nevus.

The excision margins of the tumours diminished in the whole patient population during 1988-1991 from 5 cm median, range (0.5-7.0cm) to 2 cm median, the reduction in the excision margins following the recommendations based on the results of randomised trials on surgical margins at that time.

Regional lymph node dissections were performed on 128/298 patients, 95 (74%) of them being prophylactic and 33 (26%) therapeutic in their indications. The most common site of lymph node dissection was the axilla, 54.7%, followed by the groin, 32.0%, and the neck, 11.7%. The remaining 1.6% consisted of supraclavicular or para-aortal node dissections, Table 8.

Table 8
Tumour data (Study I)

Clinical presentation of tumour at first hospital visit	Not operated on	61 (20%)
	Removed	230 (77%)
	Recurrence in scar	2 (1%)
	Metastatic disease	5 (2%)
Stage of tumour at diagnosis (AJCC 1992)	In situ	16 (5%)
	Local	271 (91%)
	Regional lymph node metastasis	8 (3%)
	Distant metastasis	3 (1%)
Nevus history prior to melanoma	Absent	29 (10%)
	Since early childhood or birth	29 (10%)
	For several years	126 (42%)
	No information	114 (38%)

The survival of the melanoma patients (n = 298) was analysed. Fifty-three (18%) patients died of melanoma and 46 (15%) of other causes; 199 (67%) of the patients were alive at the end of the follow-up period. Cumulative melanoma-specific overall survival of patients with invasive melanoma (n = 282) is shown in Figure 4.

In univariate analysis, significant prognostic markers were tumour thickness (Breslow classification), depth of tumour invasion (Clark classification), stage at diagnosis, tumour ulceration and age of the patient at diagnosis. Tumour location on the trunk was a significant factor for overall survival. Tumour ulceration and tumour location on the trunk were adverse prognostic parameters. The survival outcome was better for younger than for older patients. Age of the patient is considered as a nominal parameter, the cut-off point 53.9 years representing the median value, Table 9.

Figure 4

Cumulative melanoma-specific overall survival

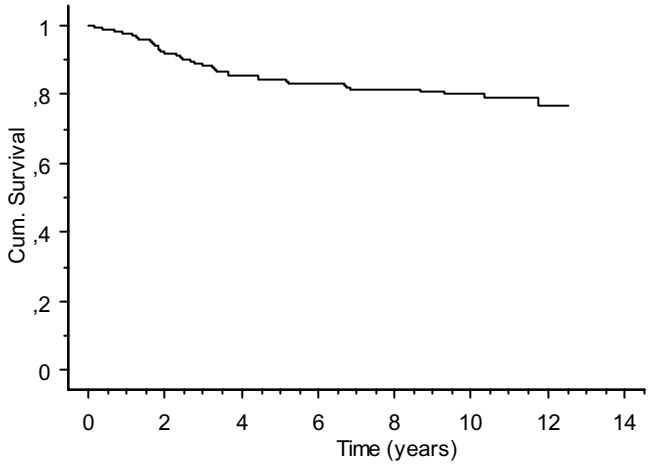


Table 9
Prognostic factors for survival, univariate analysis (Study I)

Parameter	Disease-free survival, p	Overall survival, p
Tumour thickness	‹ 0.0001	‹ 0.0001
Depth of tumour invasion	‹ 0.0001	‹ 0.0001
Stage at diagnosis	‹ 0.0001	‹ 0.0001
Tumour ulceration	0.0005	‹ 0.0002
Age at diagnosis; ≤ / > median 53.9 years	0.04	0.04
Tumour location on trunk	ns	0.03*
Gender	ns	ns
Surgical margins	ns	ns

*analysis was stratified by tumour thickness
ns = nonsignificant

In Cox multivariate analysis, tumour thickness (p = 0.01) and tumour stage (p = 0.04) were the strongest prognostic factors for disease-free survival. Tumour stage (p = 0.01), tumour thickness (p = 0.02) and tumour location on the trunk (p = 0.03) were the strongest prognostic factors for overall survival.

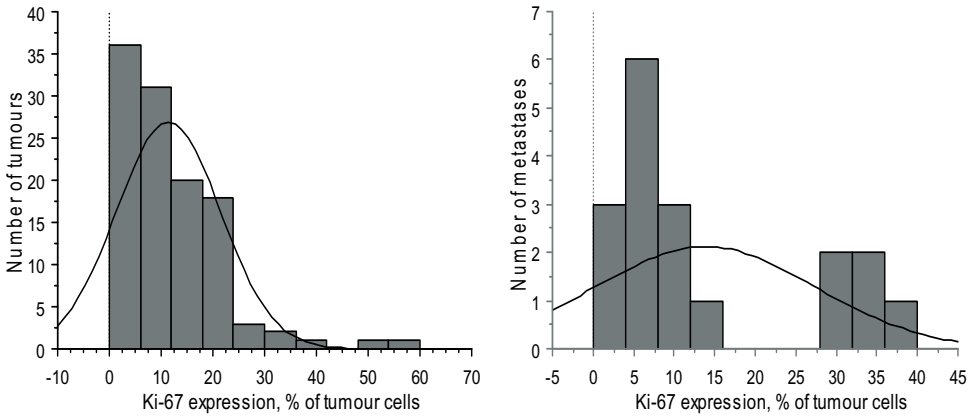
2. Study II

2.1. Immunoreactivity of Ki-67, Bcl-2 and p53 in primary and metastatic tumours

Positive immunoreactivity for Ki-67 in primary melanomas was detected in 103 tumours but was absent in ten. Positive immunoreactivity ranged from 0% to 60% in tumour cells, with a median value of 9.75%, Figure 5a. In metastatic tumours the median value of Ki-67 immunoreactivity was slightly higher (8.3% vs 7.0%) than in primary tumours, but the range was wide (0-40%), including tumours lacking Ki-67 expression, Figure 5b. Paired (18 pairs) analysis of primary and metastatic tumours of the same patient did not demonstrate any consistent trend in the behaviour of Ki-67 expression.

Figure 5
Histogram of Ki-67 expression a) in primary melanomas (n = 113) and b) in metastatic tumours (n =18)

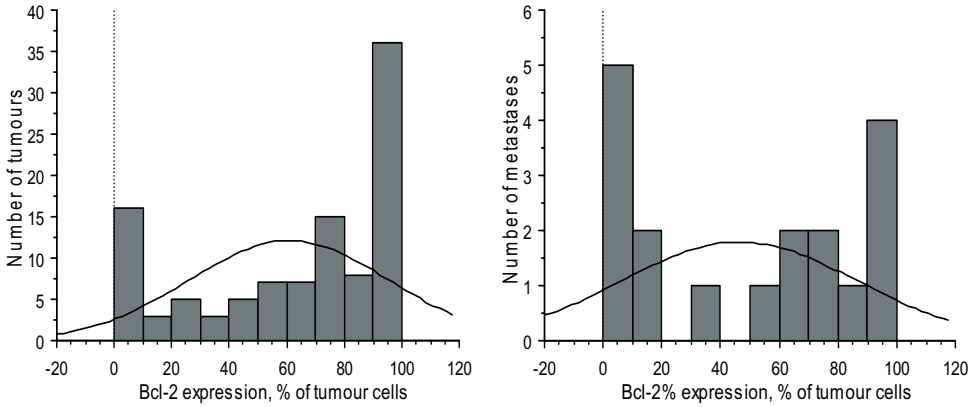
a) **b)**



Bcl-2 expression presented a wide range (0-100%) in primary tumours, with a median value of 70% of tumour cells expressing Bcl-2. The biggest subgroups of tumours were those without expression (n = 10), and those with 100% positive immunoreactivity for Bcl-2 throughout the tumour (n = 17), Figure 6a. The majority of tumours expressed Bcl-2 focally (n = 70, 67%) and 25 (24%) diffusely; 10 (9%) showed no expression. Bcl-2 expression showed a wide variation in metastatic tumour samples, too. The range of immunoreactivity was from 0% to 100% in tumour cells, with a median value of 55.0%. The biggest subset constituted tumours with less than 10% immunoreactivity, Figure 6b. Paired sample analysis of 18 cases revealed a trend towards decreasing expression of Bcl-2 during disease progression (p =0.08).

Figure 6
Histogram of Bcl-2 expression a) in primary melanomas (n = 105) and b) in metastatic tumours (n = 18)

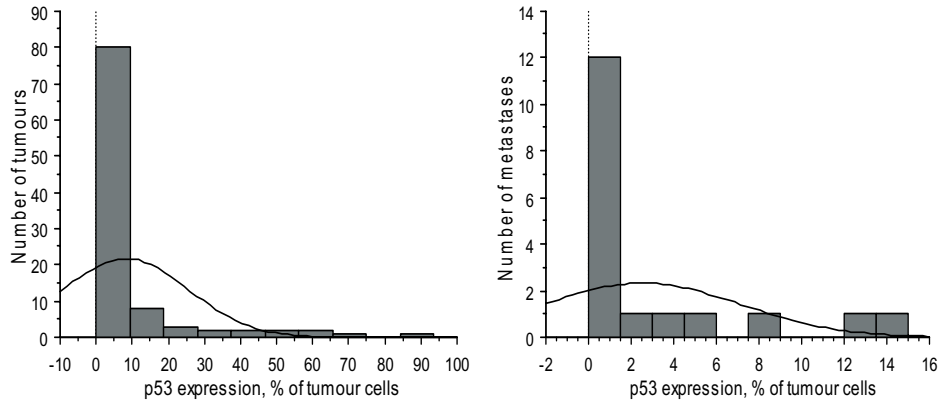
a) **b)**



In primary melanomas, 49/101 (48%) of tumours expressed no p53 at all. The range maximum for p53 expression was 94%, with a median value of 0.25%, Figure 7a. In melanoma metastases, 12/18 (67%) did not express p53. In the 6/18 (33%) of the metastases expressing p53, the level of expression was under 15%, the distribution shown in Figure 7b. Paired sample analysis of the 18 cases showed a trend towards decreasing expression of p53 during disease progression (p = 0.08).

Figure 7
Histogram of p53 expression a) in primary melanomas (n = 101) and in metastatic tumours (n = 18)

a) **b)**



2.2. Associations with clinicopathological characteristics

The Ki-67, Bcl-2 or p53 expression levels of primary melanomas were not associated with tumour thickness or tumour invasion level, nor did these markers show any correlations with each other. Ki-67 and Bcl-2 expressions were higher in ulcerative than in non-ulcerative tumours ($p = 0.12$ and $p = 0.05$, respectively). Strong Bcl-2 expression also had associations with male gender ($p = 0.04$) and old age of the patient ($p = 0.04$).

2.3. Association with prognosis

In univariate analysis of the entire patient population, neither Ki-67, Bcl-2 nor p53 were prognostic factors. However, in the subgroup of intermediate tumour thickness (Breslow 1.01 – 4.0 mm, $n = 52$), there was a trend towards disease-free survival and an overall survival benefit for those with tumours showing low Bcl-2 expression as compared with those with tumours showing high Bcl-2 expression ($p = 0.09$ and $p = 0.08$, respectively). The reason why the same association with prognosis was not discovered in thin ($\leq$ 1 mm, $n = 53$) or in thick ($\rangle$ 4 mm, $n = 11$) melanomas may be due, on the one hand, to the small number of events ($n = 4$ in DFS and $n = 3$ in OS) in thin melanomas and, on the other, to the small number of patients in the thick melanoma group. The expression pattern of Bcl-2 between absent, focal and diffuse immunoreactivity had no correlation with the clinical course of the disease.

In Cox multivariate analysis for disease-free survival, tumour thickness was the most important prognostic factor ($p = 0.0002$), and low expression of p53 indicated a favourable prognosis ($p = 0.05$). In overall survival, tumour thickness was the only significant prognostic factor ($p = 0.0006$).

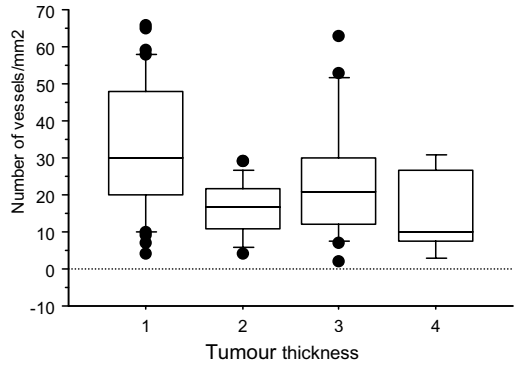
In melanoma metastasis, the level of Ki-67, Bcl-2 or p53 expression showed no association with the final outcome of the disease.

3. Study III

3.1. Immunoreactivity of CD-31 and clinicopathological associations

The blood vessel density of tumours ranged from 1 to 65 vessels/mm^2, with a median value of 20.0 vessels/mm^2. Thicker tumours showed a trend towards lower vascularisation, Figure 8.

Figure 8
Box plots of vessels detected in primary melanoma samples with different tumour thicknesses



1 = Tumour thickness ≤ 1 mm, n = 31
2 = Tumour thickness 1.01 – 2.0 mm, n = 23
3 = Tumour thickness 2.01 – 4 mm, n = 22
4 = Tumour thickness › 4 mm, n = 8

Tumour vascularity did not relate significantly to patient or tumour characteristics such as the gender or age of the patient, or the invasion level, location or ulceration of the tumour.

3.2. Association with prognosis

In univariate analysis of overall survival, the depth of tumour invasion (Clark classification) was an independent prognostic marker ($p = 0.02$) and tumour vascularity was an important, but not an independent factor determining prognosis ($p = 0.06$). High tumour vascularity was associated with a good prognosis. In Cox multivariate analysis, tumour thickness was an independent prognostic factor in disease-free survival, as was the level of tumour invasion in overall survival. Tumour vascularity held second position in both disease-free and overall survival but provided no further significant information.

4. Study IV

4.1. Immunoreactivity of tenascin-C and clinicopathological associations

The following regions of the tumours were examined by Tn-C expression: general stromal expression, stromal expression at invasion fronts and intracytoplasmic expression. The invasion regions under examination were the intraepidermal lateral border, the upper dermal lateral border and the deep, dermal or subcutaneous border.

In general, Tn-C was widely expressed in primary melanoma samples with the exception of intracytoplasmic staining, which was rare (in 20% of tumours only). The staining pattern varied from focal to diffuse in different parts of the tumour. Only in two tumours was Tn-C completely absent.

No correlation existed between intensity of Tn-C staining and tumour thickness, level of tumour invasion, tumour ulceration or the number of peritumoral lymphocytes. Neither the gender nor the age of the patient correlated with expression of Tn-C.

4.2. Association with prognosis

Absence or low expression of Tn-C in the tumour seemed to be a generally beneficial prognostic sign. The absence of Tn-C expression at the intraepidermal lateral border, the upper dermal lateral border, the deep (dermal or subcutaneous) border of the tumour or in tumour stroma of melanomas was associated with better disease-free survival of patients than was positive staining in univariate analysis ($p = 0.26$, $p = 0.06$, $p = 0.05$ and $p = 0.07$, respectively). Accordingly, the absence of intracytoplasmic staining was a beneficial prognostic sign for disease-free survival ($p = 0.04$). For overall survival, the trends were the same but not statistically significant. In multivariate Cox analysis of disease-free survival, tumour ulceration ($p = 0.01$) and intracytoplasmic expression of Tn-C ($p = 0.04$) were independent adverse prognostic factors; for overall survival, however, there were no independent prognostic factors.

5. Study V

Expression of ezrin was detected in most primary tumours (76/95, 80%). The ezrin immunoreactivity of metastatic specimens was, in general, stronger than was that of their primary counterparts; in six tumours the intensity of the metastatic tumour was higher than was that of the primary tumour, and in six tumours the intensity remained unchanged, Table 10.

Table 10
Immunoreactivity for ezrin

Specimens	Immunoreactivity for ezrin
Primary melanomas (n = 95)	
19	negative
48	weak positive
28	strong positive
Metastatic samples (n = 12)	
2	weak positive
10	strong positive

5.1. Immunoreactivity of ezrin and clinicopathological associations

The level of ezrin immunoreactivity had no association with the patient´s gender or age, or with tumour ulceration or location. However, it had a strong association with tumour thickness ($p = 0.0008$, Breslow classification) and level of tumour invasion ($p = 0.0004$, Clark classification). The number of tumours with strong ezrin immunoreactivity increased with increasing tumour thickness and level of tumour invasion. Inversely, the number of tumours with negative ezrin immunoreactivity decreased with increasing tumour thickness and level of tumour invasion. The proportion of tumours with weak positive expression of ezrin was not significantly associated with tumour thickness. There was a correlation between higher Ki-67 expression and increased ezrin immunoreactivity ($p = 0.04$). Ki-67 had a weak correlation with tumour thickness ($p = 0.10$) and a stronger correlation with level of tumour invasion ($p = 0.009$); Ki-67 being higher in thicker tumours. No correlations were found between ezrin immunoreactivity and Bcl-2 or p53 expression of tumours.

5.2. Association with prognosis

In univariate analysis, patients without ezrin immunoreactivity had a disease-free survival benefit (2/19, 10.5%, with metastatic disease) as compared with those with positive (either weak or strong) ezrin immunoreactivity (22/76, 28.9%, with metastatic disease): however, the difference was not statistically significant ($p = 0.19$). For melanoma-specific overall survival, there were no statistical differences between patients with or without ezrin immunoreactivity. In multivariate analysis for survival, ezrin immunoreactivity was not a prognostic factor.

DISCUSSION

1. Survival

The survival of melanoma patients has continuously improved in Finland. The 5-year melanoma-specific overall survival for patients diagnosed with melanoma during 1960–1969 was 51.1% and for 10-year survival 43.7%. The survival rates during 1990–1999 had, however, risen to 84.6% and 84.2%, respectively (Finnish Cancer Registry 2005). The survival of patients with localised melanoma, in particular, has improved since the early 1960s. In contrast, the survival of patients with non-localised melanoma has improved only very slowly during the same time period, the 5-year rate for patients with non-localised melanoma still being below 30% (Finnish Cancer Registry 2005). The first clinical study of a Finnish melanoma population and its survival was that of Brandt in 1956 (Brandt 1956). Half of the patients (49.7%, n = 112) already had regional or distant metastases at diagnosis, and the 5-year overall survival rate was only 20.5%. Later, in 1977, in the study of Pakkanen on a Finnish melanoma population (n = 691), 75% of men´s and 80% of women´s tumours were stage I at diagnosis, and the 10-year survival rate was 41% for men and 53% for women (Pakkanen 1977). Karjalainen et al. (1998) reported a crude 5-year survival rate of 78% for a Finnish series of 369 localised cutaneous melanoma patients between 1974 and 1989.

Our study comprised all the primary melanoma patients that were diagnosed or treated at the Department of Plastic Surgery, Helsinki University Hospital during 1988-1991. The clinical follow-up data were gathered from patients´ records, and the overall survival data indicating whether the patient had died of melanoma or of some other cause or was still alive were obtained from the National Population Registry. The reliability of the registry is considered good, although it should be noted that some information on the cause of death is probably lacking as post mortem examinations were not performed systematically on all mortalities. Further it should be noted that in the retrospective approach of this study we lacked the standardisation of treatments and follow-up as in retrospective studies in general (Altman and Lyman 1998).

Tumours were mostly localised melanomas (91%). In the whole patient population, the melanoma-specific 5-year overall survival rate was 85.7%, the 10-year OS rate 82.1%, and the 14-year OS rate 63.7%. The melanoma-specific 10-year OS rate for stage I patients (n = 153) was 92.6%, for stage II (n = 134) 72.0%, and for stage III (n = 8) 37.5%. Those diagnosed at stage IV (n = 3) died of melanoma within a few months of diagnosis. In the whole series, there were 110 (36.9%) patients with thin (≤ 1 mm) melanomas. Five (4.5%) of them developed metastases and, of them, three (2.7%) died of melanoma during the follow-up. Localised melanoma thus accounts for a high proportion of all diagnosed melanomas, and the survival rates of our

patient population are close to those documented in the Finnish Cancer Registry during the same time period.

2. Prognostic factors

Until now the most important prognostic factors have been parameters describing morphological characteristics of the tumour such as thickness, ulceration and invasion level. Additional prognostic information has been derived from patient characteristics such as gender, age and tumour localisation. The same classical parameters were also found to be important in our clinical study. Our finding of an association between melanomas arising de novo and an unfavourable prognosis should be regarded critically. Melanomas can grow slowly at the beginning and be neglected by patients until they have reached a symptomatic rapid growth phase.

The aim of primary surgery is to ensure local control of the disease. Very wide margins have not been shown to enhance prognosis (Veronesi et al. 1988, Balch et al. 1993, Cohn-Cedermark et al. 2000, Khayat et al. 2003, Thomas et al. 2004). Our study was performed at a time when a switch was being made from wide to narrower surgical margins. Our findings did not reveal any prognostic influence in the difference in surgical margins used, although the statistical power of our study, due to its relatively small patient population and its retrospective nature, is not sufficient to detect small differences in survival. As our study demonstrates, elective lymph node dissections in thick melanomas, especially in those located close to the axilla, groin or neck, were routinely performed until the early 1990s. The efficacy of these prophylactic lymph node dissections was long controversial, the procedure being associated with substantial morbidity. Recent randomised trials have shown no survival benefit in the prophylactic dissection of regional lymph nodes (Cascinelli et al. 1998, Balch et al. 2000, Lens et al. 2002).

In 1992, sentinel lymph node (SLN) biopsy (Morton et al.1992) was proposed as a minimally invasive procedure that could serve as an accurate staging method for primary melanomas carrying low morbidity for patients. The prognostic value of determining SLN status has been demonstrated in many studies (Statius Muller et al. 2001, Doting et al. 2002, Leong et al. 2005, Yee et al. 2005). We still lack evidence that removal of SLN and dissection of the regional node basin if micrometastatic melanoma is found improve overall survival. The results of ongoing multicentre trials will soon answer these questions (Morton et al. 2005).

A number of immunohistochemical, serological and molecular markers have been investigated for their prognostic value but none has so far succeeded in overcoming tumour thickness. A single mutated gene may not explain the biological character of melanoma since the process of malignant transformation is highly complicated. There is a great need for research on melanoma cells and factors related to their metastatic

potential; equally important would be research focusing on interactions in the malignant stroma. Evaluation of histological tumour markers in clinical patient series provides a means of understanding their possible role in the spread of cancers.

In our studies, we concentrated on the following parameters in our search for new prognostic information: Ki-67, Bcl-2 and p53, tumour vascularity, and expressions of the proteins tenascin-C and ezrin.

2.1. Ki-67

We found that expression of Ki-67 in primary melanoma samples was present in most tumours (103/113), the remaining tumours showing no Ki-67 expression. A cut-off point often used for Ki-67 immunopositivity of melanomas is 20% to distinguish tumours with a low proliferation rate (‹20%) from those with a high proliferation rate (≥ 20%) (Ramsay et al.1995a, Hazan et al. 2002). On that basis, only 17/113 (15%) of tumours in our series were highly proliferating, the level of positive immunoreactivity for Ki-67 presenting rather low, with a median value of 9.7% of the tumour cells. We found that the level of Ki-67 immunoreactivity did not relate to tumour thickness, in contrast to findings presented elsewhere (Moretti et al. 1990, Rieger et al. 1993, Hazan et al. 2002). Our tumour entity comprised mainly thin melanomas, 53/113 (45%) being ≤ 1 mm thick; only 11/113 (9%) were › 4 mm. This may explain the dearth of statistics showing a correlation between proliferation and tumour thickness, if such existed. Ki-67 expression was higher in ulcerative than in non-ulcerative tumours, but the correlation did not reach statistical significance. The association is of interest as the adverse impact of ulceration on prognosis in primary melanoma has now been verified (Balch et al. 2001a).

Ki-67 immunoreactivity was not a prognostic factor in our study, even if separately examined in different subsets of tumour thickness. The prognostic value of Ki-67 expression in melanomas is an unsettled issue, with the strongest associations being revealed in thick melanomas (Ramsay et al. 1995a, Boni et al. 1996, Vogt et al. 1997). In thin melanomas, the category to which the majority of our tumours belong, other, more powerful determinants most likely direct the course of the disease.

In metastatic specimens, the level of Ki-67 expression ranged from completely negative to 40% of positive immunoreactivity of tumour cells. In the paired analysis of primary and metastatic tumours of the same melanoma patients (n = 18), Ki-67 immunoreactivity increased in ten, remained unchanged in one and decreased in seven pairs during disease progression. This leads us to speculate that the amount of proliferative cells is not crucial for the metastatic capacity of the tumour.

2.2. Bcl-2

Bcl-2 was widely expressed (95/105, 91%) in our series of primary melanomas. The same has been discovered by other investigators of primary melanomas (Cerroni et al. 1995, Tron et al. 1995, Grover and Wilson 1996). Bcl-2 expression was not a prognostic factor in our study population, which included patients with a wide range of tumour thickness (Breslow 0.3-10 mm). In the subgroup of tumours of intermediate thickness (Breslow 1.01-4.0 mm, n = 52), however, Bcl-2 expression did provide prognostic information as patients with tumours showing low Bcl-2 expression demonstrated a trend towards favourable survival as compared with those with tumours showing high Bcl-expression.

Earlier incongruent results (van den Oord et al. 1994, Ramsay et al. 1995b, Tron et al. 1995, Grover and Wilson 1996) of the association of Bcl-2 expression with melanoma progression and prognosis are a subject for speculation. Low expression of Bcl-2 in primary or metastatic melanomas, or both, might be explained by acquired autonomous growth properties of the more malignant lesions, which would therefore reduce the physiological requirement for Bcl-2 to maintain the anti-apoptotic capacity of melanoma cells. Genetic alterations are suggested to decrease the dependence on Bcl-2 for cell survival, other anti-apoptotic genes playing a major role.

Our results for decreasing Bcl-2 expression in metastatic specimens support a recessive role of Bcl-2 in metastatic tumour growth. In contrast, a study by Grover and Wilson (1996) reported the maintenance of Bcl-2 expression and its association with a poor prognosis of metastatic melanoma. Moreover, the melanoma metastases of patients not responding to chemotherapy have been reported to express particularly high levels of Bcl-2 protein, supporting the concept of Bcl-2 as a key regulator for chemotherapy-triggered apoptosis in malignant melanoma (Vlaykova et al. 2002, Hakansson et al. 2003). There are studies that report promising results with Bcl-2 antisense therapy for melanoma patients, especially in combination with chemotherapy (Jansen et al. 1998, Jansen et al. 2000). It is known that susceptibility to apoptosis depends on tumour type (Staunton and Gaffney 1995); the role of Bcl-2 may also be tumour specific. Our findings indicate an adverse character for high Bcl-2 expression in primary melanoma. It is hoped that future studies will clarify the value of Bcl-2 as a predictive marker, and also its possible use in immunotherapy.

2.3. p53

We discovered that the immunohistochemical detection of p53 in primary melanomas was low, with 49% of tumours completely negative for p53 expression. Immunohistochemical staining can detect the p53 protein in only very small amounts in the normal skin (Lane and Benchimol 1990). Most studies report the absence of p53 expression in melanocytic nevi (Radhi 1999, Sauder 1999). In primary melanomas, *p53*

gene mutations are rare, amounting to less than 5%. However, the frequency of positive immunoreactivity differs (0-66%) among different studies (Florenes et al. 1994, Lee et al. 1995, Radhi 1999, Sauder 1999). In some internal cancers, *e.g.* colorectal and lung cancers, concordance exists between the presence of *p53* gene mutations and expression of the p53 protein in immunohistochemistry (Rodrigues et al. 1990, Bodner et al. 1992). In melanomas, in contrast, no such relationship exists, indicating that not all immunohistochemically detectable p53 expression is due to the presence of gene mutations but rather is a sign of disturbed cellular processes (Florenes et al. 1994, Akslen et al. 1998). In our series of metastatic samples, p53 expression was even more rare and the majority of tumours (67%) did not express it at all. Results for the presence of *p53* gene mutations in metastatic tumours vary in different studies ranging from 9% (Zerp et al. 1999) to 23% (Florenes et al. 1994) and to 70% of tumour cells (Sparrow et al. 1995).

Our findings of no association between p53 expression and tumour thickness in primary melanomas and the strongly reduced expression of p53 in metastases imply that p53 expression is an early event in tumour progression. In multivariate analysis for disease-free survival of patients with primary tumours, high expression of p53 was an independent adverse prognostic factor. Thus high p53 expression in primary melanomas may be associated with altered cell cycle regulation and thus have an effect on the course of the disease.

p53 mutations occur at a much lower frequency in primary melanomas than they do in non-melanoma skin cancers or any carcinomas, and the role of these mutations in melanocytic tumours is still unclear. Expression of p53 can obviously not be used to discriminate between benign and malignant melanocytic tumours, and the prognostic value of p53 in primary melanoma will have to be determined in further investigations.

2.4. Tumour angiogenesis

We discovered that tumour vascularisation was inversely associated with tumour thickness. The most richly vascularised tumours were found among the thinnest (‹ 1.0 mm) tumours, whereas vascularisation was lower in intermediate thickness (1.0–4.0 mm) and thick (> 4 mm) tumours. The subgroup of thick tumours was the smallest, comprising only eight cases. However, there was no association between tumour vascularisation and the level of tumour invasion level (Clark classification), this parameter probably not being sufficiently accurate to distinguish the thinnest tumours from others. By anatomical localisation, the most highly vascularised tumours were in the lower extremities, in the palm and sole of the foot, although the number of tumours in those regions was restricted to a couple of cases. Controversially, in the region of the head and neck, where there is abundant vascularity of the skin, tumours were the least vascularised. These findings lead us to propose that the vascularity of the tumour is not related to the vascularity of the surrounding skin; rather it is an intrinsic character of the tumour. There was no

relevant association between tumour vascularity and the presence or absence of tumour ulceration. The mechanisms behind the formation of ulceration of the overlying epidermis of the tumour are unclear; our findings suggest, however, that tumour vascularity is not a contributing factor.

From the prognostic viewpoint, melanoma patients with highly vascularised tumours had an overall survival benefit as compared with those with sparsely vascularised tumours. As tumour vascularisation was not significantly associated with tumour thickness, there has to be some other explanation for this result. The upper part of healthy skin is more highly vascularised than the deeper parts, and our immunohistochemical technique was not able to distinguish pre-existing vessels from the intratumoral vessels formed by tumour angiogenesis. Furthermore, it has been shown that many tumours induce a peritumoral vascular plexus that is the result of tumour angiogenesis, just like the tumoral vessels. This plexus may have a stronger impact on overall vascular counts in thin melanomas than it does in thicker and larger tumours.

The discrepancies between studies regarding the importance of angiogenesis in melanoma progression and metastasis may have arisen due to the use of patient populations not matched for clinical factors that might influence metastasis. Factors such as the capacity of a tumour for vascular invasion, the ability of a tumour to evade the immune system and the immunocompetence of the host may also vary. These cannot be standardised in prognostic studies, thus making it more difficult to evaluate the precise role of tumour vascularity. The differences in results may also be due to the range of methods applied for microvessel staining and for quantifying angiogenesis. Besides microvessel count and vascular density, other parameters including quantification of vessel area, microvessel branching and distinct vascularisation patterns have been assessed and analysed in relation to prognosis (Massi et al. 2002).

As well as immunohistochemisty, methods such as colour Doppler sonography have been used in detecting intratumoral vessels (Lassau et al. 2002). Elevated expressions of several angiogenic factors, including VEGF, bFGF, and IL-8, have been detected in primary cutaneous melanoma, and the importance of these mediators in promoting melanoma angiogenesis, metastasis and survival has been confirmed in tumour xenotransplant models and in clinical studies (Ugurel et al. 2001, Streit and Detmar 2003). Mouse model systems and clinical studies have been performed to evaluate the efficacy of angiogenesis inhibition with several types of molecules in melanoma (Kuenen et al. 2003, Reisfeld et al. 2004).

In contrast to many solid tumours that exhibit a relation between the level of tumour vascularisation and prognosis, such as in breast cancer (Choi et al. 2005), lung cancer (Singhall et al. 2005) and prostatic carcinoma (Quinn et al. 2005), in melanoma the issue is more complicated and the prognostic importance of the degree of tumour vascularisation has remained controversial. Tumour vascularisation has not yet been evaluated in large matched cohorts of patients with melanoma.

2.5. Tenascin-C

In our study, the majority of the tumours expressed Tn-C, only two out of 98 being completely negative for Tn-C. A quantative difference in the expression of Tn-C has previously been reported in benign, dysplastic and malignant tumours. All benign nevi have shown moderate expression of Tn-C at the dermoepidermal junction, but at a markedly lower level than in malignant tumours (Tuominen and Kallioinen 1994). Tn-C is not specific to cancer but its upregulation is associated with processes such as organogenesis, wound healing and invasive growth.

Our study was the first, to our knowledge, to correlate the immunohistochemical findings of Tn-C in melanomas with the prognosis for patients. Increased expression of Tn-C in the stroma of invasive regions or in the cytoplasm of malignant cells correlated with a poorer clinical course of the disease as compared with those lacking Tn-C expression, significantly so in disease-free survival. In overall survival, the difference was not marked. This finding suggests that the appearance of Tn-C in the invasive regions is a sign of an active tumour with the capacity to grow invasively and to metastasise. We did not study the expression of Tn-C with in situ hybridisation and therefore the origin of Tn-C could be in extracellular matrix and/or melanoma cells. Evidence exists, however, that in regions of tumour invasion Tn-C is produced by cancer cells (Yoshida et al. 1997). The role of intracytoplasmic Tn-C in melanoma cells, aside from invasive regions, requires further investigation. There is no evidence that intracytoplasmic or extracellular matrix Tn-C is any different in structure or function. In this study we did not examine the different isoforms of Tn-C. Intracytoplasmic immunoreactivity may be a sign of significant synthesis of Tn-C in these cells.

Clinical studies on the relevance of the level of Tn-C expression in malignant tumours and its relation to patient survival have shown inconsistent results. Tn-C most likely has different roles in different tissues. Further, its function may differ, depending on whether it is located in invasion regions or inside the tumour. Moreover, different isoforms may have different functions (Chiquet-Ehrismann and Chiquet 2003).

Clearly, Tn-C cannot be a discriminate of malignancy; rather its expression might be regarded as a sign of the invasive character of the tumour. Future studies will show whether expression of Tn-C could be used as a prognostic marker and a tool in clinical treatment decisions for primary melanoma and whether it could be used as a target for immunotherapy. As Tn-C has also been detected in the sera of patients with advanced melanoma (Herlyn et al. 1991), it has potential as a serum marker for the progression of melanoma.

2.6. Ezrin

Most of the primary melanomas (76/95) expressed ezrin in our study. Increased ezrin expression was associated with increased tumour thickness and Ki-67. Patients with high ezrin expression in primary melanomas showed a trend towards poorer disease-free survival than did those with low ezrin expression in tumours. However, in this rather small patient population the difference was not statistically significant. For melanoma-specific overall survival there were no differences between tumours with or without ezrin immunoreactivity. Expression of ezrin was noted in all the metastatic samples of 12 patients, and the intensity in metastatic tumours was higher (n = 6) than in their primary counterparts or remained unchanged (n = 6). This interesting preliminary finding will have to be evaluated with a bigger patient population.

Although we detected no significant association between ezrin expression and the prognosis of primary melanoma, there was evidence that ezrin expression is associated with tumour growth. This might be due to the involvement of ezrin in many intra- and intercellular processes that may directly contribute to the growth potential of the tumour. Another alternative is that ezrin is a marker of a melanoma cell that is responding to growth factors, adhesion molecules and other signalling pathways dependent on ezrin (Hunter 2004).

Activated ezrin possesses a central link at the cell membrane and cytoskeleton interface. It conducts signals between metastasis-associated cell-surface molecules and signal transduction components. Signals from the cell surface are passed through the Rho signal pathway or through the antiapoptotic molecule Akt, or MAPK (Hunter 2004), which are important for successful metastasis (Khanna et al. 2004). Ezrin associates with the extracellular matrix mediated by integrins and takes part in the cell-cell interaction mediated by E-cadherin, both essential areas for a malignant cell in the invasive and metastatic processes (Hunter 2004). In melanoma, MAPK activation plays a key role in radial growth phase melanoma and at later stages (Cohen et al. 2002). The Ras-Raf-MAPK pathway is activated in the majority of melanomas because of a mutation in the *BRAF* gene (Collisson et al. 2003). In an experimental study on mice with metastasised melanoma, an inhibitor of MAPK phosphorylation was introduced, resulting in rapid regression of pulmonary metastases and inhibition of the formation of new ones (Collisson et al. 2003). Similarly, a previous report showed that inhibition of MAPK signalling in melanoma cells induced apoptosis, suggesting a direct role for MAPK in signalling malignant transformation of melanocytes and in survival (Koo et al. 2002). Enhanced ezrin expression in melanoma cells may therefore contribute to increased MAPK levels and thus influence tumour cell proliferation and metastasis.

More clinical investigations on larger patient populations are awaited to enable us to further evaluate the role of ezrin in melanoma development and to determine its prognostic and possible therapeutic role in primary melanoma.

2.7. Methodological aspects

Compared with cell lines, immunohistochemistry can not directly answer to what are the background molecular mechanisms in the malignant progression of cutaneous melanoma and how the prognostic variables exert their influence on this disease (Newton Bishop 1997). However, immunohistochemistry is generally rather specific and sensitive method and is routinely performed by many pathology laboratories. It is also important to test whether the theories on the molecular mechanisms in vitro apply in clinical materials, because the expression patterns and biological properties of cell lines may change during culture (Newton Bishop 1997, Bodey 2002). The weakness of immunohistochemistry is the poor standardisation. One major cause of variation in the reproducibility of immunohistochemical staining is induced by different tissue fixation and processing. False interpretations may also be caused by tumour heterogeneity as the analysed specimen may not be representative (Bodey 2002). Studies differ in the manner in which the same antigen is quantified. For instance, tumour vascularity can be quantified by the number of vessels or by the maximum diameter of vessels per unit area of the tumour or by the percentage vessel area of the tumour area examined (Srivastava et al. 1988). Accordingly, Bcl-2 expression can be quantified by intensity or the percentage of area expression or by multiplying these to obtain staining scores (Kallio et al. 2004). All of these different parameters affect the diversity of the immunohistochemical staining results and make comparison difficult.

3. Future prospects

Evaluation of the stage and prognosis of melanoma can be expected to become more precise with the application of new technologies such as gene profiling (DNA microarray) and reverse transcription polymerase chain reaction (RT-PCR) procedures. Imaging of patients with position emission tomography (PET) has turned out to be useful in detecting occult micrometastatic disease and will achieve greater importance if effective treatment modalities are found. Ideally, both known and future tumour-related prognostic markers would select the high-risk patients who would receive more intensive follow-up and be the focus of adjuvant therapies. The majority, the low risk patients, would need less frequent or shorter follow-ups, thus reducing treatment costs. Moreover, these patients would be given a presumably favourable prognosis, which would contribute to their quality of life. Some of the known or future tumour-related prognostic factors may provide a basis for tailored melanoma therapy. Some tumour markers may also serve as predictive factors in assessing treatment response and in surveillance.

CONCLUSIONS

In our study on primary melanoma we reached the following conclusions:

1. In a Finnish population of 298 primary melanoma patients diagnosed during 1988–1991 the melanoma-specific 5- and 10-year survival rates were 85.7% and 82.1%, respectively; 91% of the melanomas were staged as local at the time of diagnosis. The most important prognostic factors were tumour thickness, level of tumour invasion, stage of tumour and tumour ulceration. Ulceration was an unfavourable prognostic marker. Younger patients had better survival outcomes than did older ones. Tumour location on the trunk was an adverse prognostic factor. In this series, surgical margins seemed to have no effect on survival.

2. High expression of p53 was associated with poor disease-free survival. Increased expression of Bcl-2 was associated with other adverse prognostic markers (tumour ulceration, male gender and old age of the patient). In intermediate-thickness melanomas, an increased level of Blc-2 showed a trend towards poor survival. Expression of Ki-67 was not a prognostic marker for primary melanoma.

3. High vascularisation of primary melanomas was associated with a better prognosis but is not an independent prognostic indicator.

4. In primary melanomas, absence of Tn-C in the stroma of invasion fronts and within tumour cells seemed to be related to more benign disease behaviour with a lower risk of developing metastases.

5. Expression of ezrin in primary melanomas correlated with tumour proliferation (Ki-67), thickness and level of invasion, suggesting an association between ezrin expression and tumour progression. In survival analysis, expression of ezrin did not reach prognostic significance.

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