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Prognostic Parameters in Uveal Melanoma: A Review

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Abstract. Histologic cell type, largest tumor diameter and tumor location have traditionally been regarded as the leading predictors of survival for uveal melanoma. Morphological cell typing is, however, subjective to variations in interpretation. More objective classification parameters have emerged from extensive cytomorphometrical and DNA flow cytometrical studies. For patients with uveal melanoma there is no effective therapy if metastases have developed, and the median survival after clinical diagnosis of hepatic metastases is extremely poor. Current research focuses on the mechanisms underlying the metastatic process, including tumor vasculature, cytogenetics, oncogene activation, immunology, melanoma-associated antigens and tumor cell migration (cell-cell and cell-matrix interaction). Several new prognostic parameters have emerged from these studies, such as closed vascular patterns, loss of one chromosome 3, and different indices of cell proliferation. Furthermore, considerable genotypical and phenotypical differences have been found between uveal and cutaneous melanoma. In prospective studies on large series of melanomas a combination of histopathological and/or clinical prognostic parameters might be selected with high sensitivity and specificity, providing a way of selecting patients at high risk of developing metastatic disease, who might be eligible for adjuvant therapy. (*Surv Ophthalmol* 41:215–228, 1996)

Key words. chromosome aberrations • genetics • immunology • melanoma • melanoma-associated antigen • metastasis • uveal melanoma

Melanoma of the uvea is the most common primary intraocular malignancy in adults. The estimated incidence is six cases per one million subjects per year in the western world. The incidence in whites is eight times that in blacks.³⁶ Uveal melanomas metastasize relatively late: the 5, 10 and 15-year survival rates based on tumor-related mortality are reported to be 72%, 59% and 53 %, respectively,^{33,48,81} compared to age-matched controls. For patients with uveal melanoma there is no effective therapy if metastases have developed. The median survival after clinical diagnosis of hepatic metastasis is extremely poor: between two¹¹⁶ and seven months.⁶⁷ Survival time for uncured patients is related to the rapidity of the metastatic process. In order to lower melanoma-related mortality, it is

essential to prevent or eradicate metastatic disease. This calls for early detection and for the development of reliable prognostic factors. It is, therefore, necessary to increase our knowledge of the mechanisms underlying metastasis and the identification of reliable progression parameters as prognostic markers in primary uveal melanoma.

Although uveal and cutaneous melanoma are from similar embryological origin, they differ in biological behavior. Choroidal and ciliary body melanomas metastasize hematogeneously and preferentially first to the liver, whereas cutaneous melanomas spread to regional lymph nodes. There are no demonstrated lymphatics within the uveal tract or in the posterior orbit.

In view of the many factors involved in the pro-

TABLE 1

Abbreviations Used in this Review

Agnor:	Silver staining of the nucleolar organizer region
CAM:	Cellular adhesion molecule
Cdk:	Cyclin-dependent kinase
CEA:	Carcino embryonic antigen
CK:	Cytokeratin
EGF:	Epidermal growth factor
ELAM:	Endothelial leucocyte adhesion molecule
FCM:	Flow cytometry
FNAB:	Fine needle aspiration biopsy
Gy:	Gray
HLA:	Histocompatibility antigens
HMW:	High molecular weight
HPF:	High power field
ICAM:	Intercellular adhesion molecule
Ig:	Immunoglobulin
IL:	Interleukin
kD:	Kilodalton
LFA:	Leucocyte function-associated antigen
LTD:	Largest tumor diameter
MAA:	Melanoma-associated antigen
MAb:	Monoclonal antibody
MLN:	Mean of the largest nucleoli
mRNA:	Messenger RNA
NCAM:	Neural cell adhesion molecule
NOR:	Nucleolar organizer region
PA:	Plasminogen activator
PCNA:	Proliferating cell nuclear antigen
PCR:	Polymerase chain reaction
SDNA:	Standard deviation of the nucleolar area
S-phase:	Synthesis phase
TGF:	Transforming growth factor
TIL:	Tumor-infiltrating lymphocytes
TNF:	Tumor necrosis factor
t-PA:	Tissue-type plasminogen activator
u-PA:	Urokinase-type plasminogen activator
VCAM:	Vascular adhesion molecule
VLA:	Very late activation antigen

cess of metastasis it seems appropriate to provide an update on research on progression parameters as prognostic markers and to relate these factors to findings on cutaneous melanoma. Table 1 defines the abbreviations that will be used throughout this review.

Clinical and Histopathologic Prognostic Parameters

CLINICAL PROGNOSTIC PARAMETERS

The clinical symptomatology of uveal melanoma depends on the location of the tumor: tumors of the posterior pole cause early symptoms. They can be diagnosed by indirect ophthalmoscopy and A and B mode ultrasonography, and evidence of growth can be established by serial examinations with these methods. Computed tomography and magnetic resonance imaging are employed in diagnostic evaluation, but they have not replaced ul-

trasonography as the most accurate method of substantiating the diagnosis and determining tumor size.¹¹⁹ The A-scan provides accurate information regarding internal reflectivity and the B-scan provides two-dimensional topographic information. Tumor height can be followed for growth by B-mode ultrasonography and tumor diameter can be evaluated by serial fluorescein angiography. The clinical tumor size is defined as small (3 mm or less in thickness and smaller than 10 mm diameter), medium (between 3 and 5 mm in thickness and/or between 10 and 15 mm in diameter), or large (greater than 5 mm in thickness and/or greater than 15 mm in diameter).¹¹⁹ In a recent clinical analysis study of small choroidal melanocytic tumors it has been shown that documented growth of these small tumors increased the risk for metastases almost eight times more than a non-growing tumor.¹²¹ Therefore, active treatment rather than observation was recommended for those precursor lesions. High risk clinical factors predictive of tumor growth included greater tumor thickness, posterior tumor margin touching optic disk, subretinal fluid, symptoms of flashes, floaters, blurred vision and orange pigment.¹²¹

Additional clinical prognostic parameters include a larger tumor diameter,^{43,116} presence of extrascleral growth, tumor margin location anterior to the equator of the eye,^{83,84,116} older age,⁴³ male gender,^{42,43,74} and tumor-induced glaucoma,¹⁹ which are associated with a poorer life prognosis. The association of ciliary body involvement (location of the anterior tumor margin) with tumor-related mortality has been shown to be primarily due to ciliary body tumors being larger ($p=10^6$) with more malignant cytology ($p=3 \times 10^5$).⁸³ A comparative analysis of melanoma reports between 1966 and 1988 indicated that the combined weighted estimates of 5-year mortality rates following enucleation were 16% for histologically small tumors (LTD <10 mm), 32% for medium (LTD 10–15 mm), and 53% for large (LTD >15 mm) tumors.³³

HISTOPATHOLOGIC PROGNOSTIC PARAMETERS

Uveal melanomas have a spectrum of cell types, ranging from thin and plump spindle cells to epithelioid cells. In 1931 Callender developed a cytologic classification of uveal melanomas.⁹ The following types of melanoma cells were recognized: spindle A cells, spindle B cells and epithelioid cells. Spindle cell tumors tend to grow in a compact cohesive fashion, and they generally have a dense framework of reticulin fibers. Epithelioid cells grow less cohesively than spindle cells and are not surrounded by a network of reticulin. Melanomas of the mixed cell type are composed of a mixture

of epithelioid and spindle cells. This classification has appeared not to be sufficiently reproducible.⁵⁰ McLean et al demonstrated that spindle cells include a spectrum of benign and malignant cells and they proposed a modification of the Callender classification: spindle cell malignant melanomas, mixed cell melanomas and epithelioid cell melanomas.^{85,87} They also demonstrated that all melanomas containing epithelioid cells had more than 50% chance of metastatic spread, but when the size of the epithelioid cells was small the prognosis was not quite so bad. Although important trends have emerged from retrospective prognostic studies, such as relevance of size and cell type, Markowitz et al noted the absence of information on study design and methods in 50% of 76 melanoma studies from 1966 to 1988.⁷⁸

In recent studies the presence or absence of any epithelioid cells (spindle cell melanoma versus a combination of mixed cell type and epithelioid cell tumors) has been preferred.^{19,42,44,45,93,95} The biological behavior of uveal melanoma (tumor grade) depends not only on the cell type but also on several other factors, including LTD, especially LTD in contact with sclera.^{43,116} LTD ≤ 12 mm and less than two epithelioid cells per high power field (HPF) is associated with a favorable outcome.¹¹⁶ By univariate analysis, cell type and LTD share a similar level of correlation with death from uveal melanoma; however, only a limited correlation between cell type and LTD could be demonstrated.^{19,45}

Recent investigations have suggested that the presence of vascular networks, defined as at least three back to back closed vascular loops, is a feature strongly associated with death from metastatic melanoma ($p=0.0001$),^{42,43,103,113,114} followed by LTD.⁴³ Other significant factors in the Cox proportional hazard model in one study included (in descending order of importance), mitotic rate, the parallel with cross-linking vascular pattern, age, the presence of tumor-infiltrating lymphocytes (TIL),⁴³ and male gender.^{42,43} Cell type⁴² and tumor location^{42,113} did not appear to be an important prognostic parameter in the stepwise modeling. It was also demonstrated that, regardless of location (ciliary body or choroid), the presence of vascular networks shortens survival ($p=0.0001$)¹¹³ and that the aggressive behavior of ciliary body melanomas appears to be related to the tendency for vascular networks to develop in this location.¹¹³

PARAMETERS OF CELL PROLIFERATION

Indices of proliferation have retrospectively been studied in uveal melanoma, comparing their prognostic value with clinicopathologic param-

eters. Methods for assessing cellular proliferation include: 1) mitotic count, 2) DNA synthesis (S) labeling (thymidine labeling, bromodeoxyuridine incorporation) and flow cytometry by estimating the DNA S-phase fraction, 3) immunohistochemical methods, and 4) assessment of nucleolar organizer regions.

Mitotic Count

The identification of mitoses can be very difficult: only those cells that are unequivocally in mitosis are acceptable for counting. When the number of mitoses per high power field is counted, the area of the high power field should be given, preferably the number of mitoses per 1000 cells. For uveal melanoma the mitotic rate appeared to be of prognostic value in several studies.^{43,71,93}

DNA Synthesis Labeling

Quantitation of DNA can be performed by flow cytometry. A laser beam is used to quantify the fluorescence of nuclear material in suspension, counting between 10,000 and 200,000 cells, plotting the cells at the different stages of the cell cycle on a histogram. Euploid tumors contain a normal amount of genetic material, either 2c (diploid) or 4c (tetraploid). Doubling of the genetic information (DNA) occurs during the S-phase of the cell cycle. A high S-phase fraction reflects a high proliferation rate. For uveal melanoma, a significant correlation has been found between a high S-phase fraction and epithelioid cell type,⁶⁶ which is in line with earlier findings that spindle cell melanomas have lower cell turnover rates than epithelioid cells.¹¹⁰ A correlation between higher S-phase fractions and large tumors has also been demonstrated,⁶⁶ in contrast to earlier findings.¹¹⁰

The introduction of a thymidine analogue, bromodeoxyuridine, incorporated only during the synthesis phase of the cell cycle has simplified the problem of differentiating cycling from noncycling cells. The bromodeoxyuridine uptake is related to the number of cells in the synthesis phase. Thymidine incorporation into DNA during S-phase in dividing cells also provides information on cell cycle variables.¹¹ A strong correlation between bromodeoxyuridine uptake defined cell cycling and uveal melanoma-associated metastases has been demonstrated.⁷¹ Furthermore, destruction of the reproductive integrity of uveal melanomas, which received 20 Gy irradiation prior to enucleation has been demonstrated.¹¹ The methods described above need specialized equipment, are time-consuming, and are not applicable in daily routine.

Immunohistochemical Assessment

Immunohistochemical assessment of proliferative activity is easy to apply, relatively inexpensive, applicable on paraffin sections, and is probably a better marker of proliferation than determination of S-phase fraction.⁶⁶ Applicable monoclonal antibodies include Ki-67/Mib-1 and cyclin/proliferating cell nuclear antigen (PCNA). Multivariate analysis revealed that the Mib-1 (Ki-67) defined proliferative index is a useful independent prognostic parameter for uveal melanoma.⁹³ The Ki-67 defined proliferative activity⁹² and PCNA-defined proliferative activity¹⁰² were found to be decreased after irradiation of uveal melanomas prior to enucleation. The prognostic value of PCNA remains to be established.

Nucleolar Organizer Regions

Nucleolar organizer regions (NORs) are outpouchings of nucleolar DNA, that direct ribosomal RNA transcription. Silver staining of the nucleolar organizer regions (AgNORs) visualizes the size and number of nucleolar organizer regions as black dots, seemingly reflecting or predicting cellular proliferation. Application of this method to a series of uveal melanomas disclosed a correlation of AgNOR counts with mitoses ($p < 0.007$) and tumor size ($p < 0.009$). However, the prognostic value of AgNORs for uveal melanomas remains to be established.⁷⁷ This method is time-consuming, but neither technically demanding nor expensive.

Morphometry and Flow Cytometry

The interobserver error of the conventional Callender classification is large.^{46,50} The original Callender system was simplified in order to reduce the number of categories, which has improved the correlation of histologic features with malignant behavior.⁸⁵ However, morphological classification schemes are inherently arbitrary and subject to variations in interpretation.⁸⁴ Subsequently, a semi-quantitative system was developed to estimate the percentage of epithelioid cells in each tumor,¹¹⁶ the number of epithelioid cells and the inverse standard deviation of the nucleolar area.¹¹⁷ Furthermore, it has been shown that measurements of LTD made from glass microslides correlate with direct measurements taken from the cut surface of the globe at the time of gross examination.⁴¹ Since this refined assessment remains subjective, methods have been devised to measure cytologic features objectively.

MORPHOMETRY

Morphometry is a reliable method for objective quantification of nuclear and nucleolar character-

istics but, like DNA synthesis labeling, needs specialized equipment, is time-consuming and cannot be applied in daily routine. One of the early methods developed was measuring the standard deviation of nucleolar and nuclear features, which confirmed the value of nuclear pleomorphism for predicting the malignant potential of uveal melanomas.⁴⁷ The standard deviation of the nucleolar area (SDNA) proved to be a useful measure of the malignancy of intraocular melanomas, especially when coupled with LTD.^{49,58} The standard deviation of both nucleolar area and circumference correlated highly with survival: the nucleolar area was usually greater and more pleomorphic in the more malignant type of uveal melanoma (epithelioid or mixed versus spindle cell tumors). Unfortunately, this method requires an elaborate specially designed system. A simpler system was developed to calculate the mean of the diameters of the 10 largest nucleoli (MLN) encountered in a strip 5 mm long and one oil immersion field wide through the center of the tumor.^{45,58}

A comparison of prognostic covariables for uveal melanoma revealed that LTD, Callender cell type, SDNA and MLN correlated equally with tumor related death. MLN can be measured more easily than SDNA and is more reproducible than cell type and is therefore a useful prognostic parameter.⁸⁰ Multivariate analysis revealed that adding LTD as a prognostic covariate to either cell type, SDNA, or MLN yields a substantial increase in prognostic value.^{80,81} In contrast, a recent study demonstrated that, using multiple Cox regression models, MLN failed to exert any effect on outcome after enucleation. These authors found the presence of closed vascular loops to be the most statistically dominant histologic prognostic characteristic.¹⁰³

Recently, syntactic structure analysis has been applied to uveal melanomas. This is a mathematical method describing the basic structural units such as cells, glands and vessels as a network.¹⁶ This method seems mainly of scientific interest.

FLOW CYTOMETRY

Flow cytometry can be used to analyze the DNA and RNA content of cells. DNA flow cytometry is a reliable quantitative method of determining the nuclear DNA content (ploidy) of a tumor cell sample. Deviation from the normal amount of DNA (diploid) is known as aneuploidy. There is increasing evidence for a variety of neoplasms that DNA aneuploidy may correlate with poor life prognosis. Aneuploidy may reflect a genetically more unstable cell population with an enhanced ability to metastasize. A significant correlation between aneuploi-

dy and epithelioid cell type has been demonstrated.⁹⁵ For uveal melanoma it has been shown that ploidy abnormalities correlate more closely with survival than the standard histologic parameters,^{11,66,88,95} except in one study.¹⁷

DNA quantitation can also be carried out by static image analysis, usually by measuring the amount of light transmitted through tumor cell nuclei in a Feulgen-stained specimen. For uveal melanomas it has been found that all spindle A cells are diploid and most tetraploid peaks are formed by epithelioid cells.^{17,18} For uveal melanoma this method, however, does not appear to have additional prognostic value.⁸⁶

Nucleoli are active sites of RNA synthesis, and a relationship between nuclear RNA content and prognosis was investigated by flow cytometry using acridine orange, a metachromatic fluorochrome: the nuclear RNA content appeared to be an independent prognostic indicator in uveal melanomas, and increased RNA content was associated with a worse life prognosis.¹³

Genetic Abnormalities

CYTOGENETICS

The association of consistent chromosomal aberrations with particular types of cancer has led to the identification of some of these genes and the elucidation of their mechanism of action. The common tumor chromosome aberrations are generally classified as structural or numerical. Structural alterations include translocations, inversions, deletions, insertions, and amplifications, whereas numerical abnormalities are losses or duplications of whole chromosomes.

For cutaneous malignant melanoma and cell lines, frequent involvement of chromosomes 1, 6, 7, 9 and 11 in structural aberrations has been reported.^{69,100,108,128,130} It has been suggested that chromosome 9 plays a role during the development of cutaneous malignant melanoma, whereas chromosomes 2,3 and 6 are most likely associated with progression.¹⁰¹

Cytogenetic studies of uveal melanomas revealed that monosomy of chromosome 3 and multiplication of chromosome 8q do not occur randomly in uveal melanoma.^{26,55,56,105,106,122,136} Monosomy 3 and gain of 8q have exclusively been demonstrated in ciliary body melanomas,^{26,106} and may therefore be associated with a subgroup of uveal melanomas with poor prognosis.¹⁰⁵ Recently, it has been demonstrated that monosomy 3 is the most significant parameter ($p < 0.0001$) for poor prognosis in uveal melanoma, followed by tumor location and diameter.¹⁰⁷ Furthermore, in primary uveal melanomas,

aberrations of chromosome 6 (loss or gain) have been described.^{30,52,105,122} In cell lines obtained from metastatic uveal melanoma no consistent chromosomal aberrations have been demonstrated.⁷⁵

ONCOGENE ACTIVATION

The genetic damage found in cancer cells comprises two different categories: 1) dominant mutations which can activate proto-oncogenes to become oncogenes, or 2) recessive mutations in target genes known as tumor suppressor genes or anti-oncogenes. They are called recessive because both copies must be inactivated for tumor formation to occur.

It is now generally accepted that molecular alterations in oncogenes and tumor suppressor genes are responsible for the conversion of a normal cell into a tumor cell. Usually these genes exert normal functions in cell proliferation and differentiation.⁵

Unlike other tumors, little research has been carried out to identify and analyze the role of oncogenes and tumor suppressor genes in the development and prognosis of choroidal melanoma. Several oncogenes, including *c-myc*, have been mapped to 8q.^{128,129} The expression of *c-myc* oncoprotein has immunohistochemically been investigated in uveal melanomas and compared with other prognostic factors.^{93,111} Positive staining for *c-myc* protein correlated with proliferative index in diploid tumors, and with HMB-45 staining, but not with cell type.¹¹¹ In a study on prognosis, the percentage of *c-myc* positive cells was associated with tumor-related death. Furthermore, a strong correlation between *c-myc* and LTD was found.⁹³

Point mutations in the *N-ras* gene occur in cutaneous malignant melanomas. In patients with the *N-ras* mutation, sun exposure could have been the etiologic agent of these melanomas.¹³¹ For uveal melanomas *N-ras* mutations were not detected in two series of uveal melanoma,^{91,124} and only one *N-ras* codon 61 mutation in another series.³⁷ No H- and K-*ras* mutations were found.^{37,124} The expression of the oncoproteins *c-neu* (*c-erb-B2*: epidermal growth factor receptor) and *ras* were also analyzed with specific MAbs in a small series of uveal melanomas: in one metastasizing melanoma marked expression of *ras* was obvious.⁷⁰

Recent evidence suggests that mutation of the *p53* suppressor gene (named for the protein it encodes, *p53*) is one of the most common abnormalities found in human cancers.⁷² This gene is located on the short arm of chromosome 17 and encodes a 53 kD nuclear protein that appears to be involved in regulating the cell cycle. The nor-

mal p53 product has been shown to act as a tumor suppressor, but various point mutations within the coding region of the gene inactivate or alter this function. These mutations arise relatively late in neoplastic progression and may correlate with malignant transformation. However, in order to evaluate p53 as a diagnostic marker, correlations between p53 mutations (DNA sequence), protein overexpression (immunocytochemistry positivity), and tumor behavior need to be considered.¹³⁷ For cutaneous melanoma significantly increased prevalence of mutant p53 was found in metastatic melanoma, compared to primary tumors.¹²⁶ In contrast, other investigators found that the p53 staining was not correlated to subsequent development of local metastases, and a significant decrease of p53 protein in metastatic lesions was found in comparison to the corresponding primary tumors.² Staining for mutant p53 protein expression was found in 12 out of 18 primary uveal melanomas, whereas choroidal nevi were negative. In two melanomas expression of the p53 protein was confirmed by the demonstration of mutations in exon 7. These observations suggest that acquisition of abnormalities of the p53 gene may be an important step in the progression of uveal melanoma.¹²⁷ Recently, mutant p53 has been demonstrated in museum specimens of a family with a history of four generations of uveal melanoma associated with breast cancer.⁶⁰

Recently, homozygous deletions of a gene (Multiple Tumor Suppressor 1) have been reported in a wide variety of tumors, including cutaneous melanoma. This gene encodes a protein, previously identified as inhibitor (p16) of an enzyme called cyclin-dependent kinase 4 (Cdk4), which is involved in the cell division cycle.⁶³ Currently, uveal melanoma cell lines are under study.

Examination of mRNA levels of 21 different oncogenes, anti-oncogenes, growth factors and proteases in 17 cutaneous melanoma cell lines revealed a significant correlation between c-myc, p53 and c-src-1 (tyrosinase-kinase) levels, and between p53 and c-erb-B2 (EGF-receptor), which may be due to common regulatory control of these genes in cells of the melanocytic lineage.¹⁴ This is in accordance with the view that oncogenic transformation is a multistep process, involving activation of at least two genes.⁵⁷

So far no single gene has been shown to participate in the development of human cancers. For uveal melanoma, research in the field of molecular genetics has been limited. Further identification of tumor suppressor genes or oncogenes that participate in the cell cycle of uveal melanoma seems necessary.

Immunology

Cutaneous and uveal melanoma are considered to be relatively susceptible to immunologic influences because of reports of spontaneous regression⁶¹ and because of the delayed appearance of metastatic disease, sometimes decades after enucleation.²⁴ Of uveal melanomas 5–12% have tumor-infiltrating lymphocytes (TIL).³⁵ The number of cells is variable (0.1–29%), but usually low (mean 4.5%).⁸⁹ Immunohistochemical analysis of TIL revealed that T cells predominate in 74%, usually scattered, whereas B cells constitute a minority, usually present in clumps.^{31,135} This was similar to the findings by flow cytometric analysis of TIL.³⁵ The presence of more than 100 TIL per 20 HPF in uveal melanoma is associated with a decreased survival rate^{25,43} and T-lymphocytic infiltration was associated with death due to metastasis.¹³⁵ This is the opposite of what is observed with most solid tumors in adults, where the presence of TIL is associated with increased survival. It has been speculated that dissemination of tumor cells is required for the generation of a T lymphocyte-mediated immune response. Because the eye lacks lymphatic drainage the primary antigen processing is in the spleen.³⁵ Without lymphatic drainage uveal melanomas may be less likely to disseminate than most neoplasms.^{81,135}

Expression of the histocompatibility antigens HLA class I and II on neoplastic cells may be important in the host-tumor interaction. HLA class I antigen is found in most primary cutaneous melanomas and HLA class II (HLA-DR) is associated with increased thickness and early metastasis of cutaneous melanoma.⁵⁴ Class I and II antigen expression is more pronounced in the presence of a lymphocytic infiltrate.¹¹² For uveal melanoma it has been found that low levels of HLA expression (and therefore a lack of presentation of tumor-specific antigens) may lead to a low level of tumor infiltrate.³¹ Furthermore, in a murine model a considerable variation has been found in the susceptibility of human uveal melanomas to natural killer cell-mediated cytotoxicity. Susceptibility was closely related with reduced expression of MHC class I antigen expression. Disruption of natural killer cell function significantly increases the development of hepatic metastases from uveal melanoma cells.⁷⁶

Melanoma-Associated Antigens

Immunohistochemistry is now commonly used by pathologists in the differential diagnosis of anaplastic tumors. S-100, HMB-45 and NKI-C3 are examples of monoclonal antibodies (MAbs), which recognize melanoma-associated antigens (MAA).

TABLE 2

Melanoma-Associated Antibodies Expressed on Choroidal Melanomas, Assessed on Paraffin Embedded Tissue

MAb	Specificity	% Les.	% Cells	Reference
S-100	Monoclonal	90%	5–100%	Burnier ⁸
		20%*	>75%*	Burnier ⁸
S-100	Polyclonal	97%	n.s.	Kan-Mitchell ⁶⁵
MAb079	S100α/β	15%	n.s.	Kan-Mitchell ⁶⁵
HMB-45	100 kD	96%	n.s.	Kan-Mitchell ⁶⁵
HMB-45	100 kD	100%	5–100%	Burnier ⁸
		95%*	>75%*	Burnier ⁸
HMB-45	100 kD	99%	5–100%	Steuhl ²⁵
		77%*	>50%*	Steuhl ²⁵
NKI-C3	gp90-34	81%	>10%	Ringens ¹⁰⁹
ME491	HMW	87%	5–100%	Folberg ⁴⁰
MAb8-1H	40–50 kD/O-MAA	n.s.	n.s.	Donoso ³⁴
4A3	55–60 kD/O-MAA	n.s.	n.s.	Domato ²⁷

MAb: monoclonal antibody. % les.: percentage positive stained lesions; % cells: percentage positive cells. n.s.: not specified. *: sensitivity. kD: kilodalton. HMW: high molecular weight. O-MAA: ocular melanoma-associated antigen.

These antibodies are routinely applied in daily practice to differentiate melanomas from other tumors in routinely fixed, paraffin-embedded tissue (Table 2). With antisera generated against the S100 protein, immunoreactivity was noted in 90–97% of primary choroidal melanomas.^{8,65} In contrast, only 15% of the tumors stained with a MAb specific for both S100α and S100β, whereas 85% of all cutaneous melanomas are stained with this antibody. These findings suggest the possibility that a variant S100 protein exists in choroidal melanoma.⁶⁵ This has been confirmed at the mRNA level by a quantitative PCR method: choroidal melanomas expressed little or no S100β.⁶⁴ Although quite sensitive, antibodies to S100 are not melanoma-specific. The MAb HMB-45 labels a cytoplasmic antigen produced by fetal melanocytes and melanoma cells of adults⁵¹ and is specific and highly sensitive for cutaneous melanoma and junctional nevi in formalin-fixed, paraffin-embedded tissue.⁹⁹ It recognizes an antigen that is expressed by stimulated melanocytes.¹²³ More than 95% of choroidal melanomas express the HMB-45 antigen (Table 2).^{8,65,125} The HMB-45 antigen is immuno-electron-microscopically found in melanosomes at stage II and III. This leads to the conclusion that proliferating melanocytes express the antigen.¹²⁵ Benign proliferative cells cannot be distinguished from malignant proliferating cells with the aid of this antibody. Burnier et al found that expression of HMB-45 appeared to be greater in active uveal nevi than in inactive nevi.⁸

The MAb NKI-C3 (gp 90–34) also recognizes a melanoma-associated antigen and was reported to be positive in 81% of choroidal melanomas.¹⁰⁹ The antigens recognized by MAb NKI-beteb (gp 100) are localized at the inside of premelanosomal ves-

icles.¹³² This MAb has a striking similarity to HMB-45, but in addition recognizes resting adult melanocytes in skin.¹

Interestingly, MAb CAM 5.2 directed against intermediate filaments cytokeratins (CK) 8 and 18 reacted more often with formalin-fixed, paraffin-embedded primary uveal melanomas (38%) than with their cutaneous counterpart (6–14%).⁴⁴

Several other MAbs against uveal and cutaneous MAAs have been assessed on choroidal melanomas (Table 2).^{27,34,40} Many primary malignant tumors, including uveal and cutaneous melanomas, that initially appear homogeneous by conventional light microscopy actually consist of heterogeneous groups of cells displaying different biochemical or antigenic properties. A light microscopic feature of some uveal melanomas is the presence of well-localized, morphologically distinct areas within the same tumor. These have been attributed to separate clones of cells in different growth phases. Such cells might be expected to have different and distinct antigenic and cytomorphologic characteristics.³⁴

In order to evaluate the differences and similarities in antigenic expression patterns between cutaneous and uveal melanomas, a panel of MAbs directed against cutaneous MAAs has been applied on frozen sections of choroidal melanomas. Table 3 represents the markers expressed by the majority of uveal melanomas. MAbs NKI/C3 and NKI/beteb had a high sensitivity for choroidal melanomas in frozen sections.^{10,104,109} Although marked variation of antigen expression in uveal melanoma was noted, certain patterns of antigen expression within an individual lesion were present.¹⁰⁴ A number of MAbs (NKI-beteb, NKI-C3, M-2-2-4, Pal-M196

TABLE 3

Markers Expressed on the Majority of Choroidal Melanoma as Recognized by MAbs Raised Against Cutaneous Melanomas

MAB	Specificity	% Les.	% Cells	Reference
NKI-beteb	100 kD	86%	>50%	v.d.Pol ¹⁰⁴
NKI-beteb	100 kD	100%	>70%	Carrel ¹⁰
NKI-beteb	100 kD	100%	>60%	Ringens ¹⁰⁹
NKI-C3	gp90-34	100%	>60%	Ringens ¹⁰⁹
NKI-C3	gp90-34	87%	~100%	Carrel ¹⁰
Pal-M1	tr. rec.	n.s.	<70%	v.d.Pol ¹⁰⁴
Pal-M1	tr. rec.	75%	20-80%	Carrel ¹⁰
M-2-2-4	diff. ag.	37%	5-100%	v.d.Pol ¹⁰⁴
M-2-2-4	diff. ag.	87%	>80%	Carrel ¹⁰
G7E2	gp120-110	87%	30-100%	Carrel ¹⁰
225.28S	HMW	n.s.	<70%	v.d.Pol ¹⁰⁴
225.28S	HMW	50%	<50%	Natali ⁹⁷
G7A5	HMW	75%	>80%	Carrel ¹⁰
AMF-6	HMW	n.s.	n.s.	v.d.Pol ¹⁰⁴
AMF-6	HMW	50%	>70%	Carrel ¹⁰
AMF-6	HMW	17%	n.s.	Ringens ¹⁰⁹
AMF-7	MA-CAM-1	17%	5-100%	v.d.Pol ¹⁰⁴
AMF-7	MA-CAM-1	63%	~100%	Carrel ¹⁰
CL 203.4	ICAM-1	87%	>50%	v.d.Pol ¹⁰⁴
CL 203.4	ICAM-1	42%	5-100%	Natali ⁹⁷
Pal-M2	gp-95	75%	20-80%	Carrel ¹⁰
Mel 14	gp33-38	68%	5-100%	Carrel ¹⁰
Mel4/D12	gp33-38	68%	5-100%	Carrel ¹⁰
R24	GD3	50%	5-100%	Carrel ¹⁰

MAB: monoclonal antibody. % les.: percentage positive stained lesions; % cells: percentage positive cells. kD: kilodalton. gp: glycoprotein. Tr. rec.: transferrin receptor. Diff. ag.: differentiation antigen on melanomas and nevi. HMW: high molecular weight. MA-CAM: melanoma-associated cellular adhesion molecule. ICAM: intercellular adhesion molecule. n.s.: not specified.

and G7E2) stained uveal melanomas as well as cutaneous melanomas (Table 3).¹⁰

Of a panel of 18 MAA-MAbs applied on uveal melanomas, the expression of nine MAbs (TP39.1, TP36.1, 345.134, M111, CL203.4, M2590) was similar to that in cutaneous melanomas.⁹⁷ The studies revealed that the high molecular weight (HMW) antigen and ganglioside antigens were markedly less expressed on uveal melanomas than on cutaneous melanomas.^{10,97,104} The prognostic value of these markers remains to be established, although preliminary findings indicate that expression of NKI-beteb is related to a favorable prognosis in uveal melanoma (v.d. Pol unpublished data).

HLA class I stained 75-85% of the uveal melanomas.^{10,89,97} No correlation has been found between HLA class I expression and uveal melanoma cell type.^{59,89} Others observed an absence of class I expression on pure spindle melanoma,⁹⁷ or, in contrast, expression of HLA class I to predominantly spindle cells.¹⁰ HLA class II was detected in a limited number of lesions.^{10,59,89,97} The prognostic val-

ue of HLA class I and II in uveal melanoma is currently under study. Uveal melanomas that had been irradiated with 2x 4 Gy before enucleation had a significantly lower lymphocytic infiltrate and a significantly lower expression of HLA class II.⁵⁹

It was concluded that the overall surface antigen phenotype of the uveal melanomas tested differs markedly from that of cutaneous melanomas as defined by the panel of MAbs directed against cutaneous MAAs.¹⁰ This might suggest a different origin of the normal melanocyte giving rise to uveal tumors.¹⁰

Cell-Cell and Cell-Matrix Interaction

CELL-CELL INTERACTION

Cell adhesion molecules (CAM) are transmembrane proteins, connecting the cytoskeleton with the extracellular matrix. They have been implicated not only in intercellular recognition, but also in morphogenetic events, regeneration, tumor invasion and metastasis. One CAM binds either to another identical CAM molecule on an opposing cell (homophilic binding) or binds to a receptor molecule of different identity (heterophilic binding). Of these adhesion molecules four families can be recognized: the immunoglobulin superfamily, the cadherin family, the integrin superfamily and the selectin family.^{6,7}

The *immunoglobulin (Ig) superfamily* contains a series of CAMs which mediate Ca²⁺-independent cell adhesion. Most members of this family are single-path transmembrane glycoproteins, which act by homophilic recognition. Several subfamilies can be distinguished: i.e., the neural cell adhesion molecule (NCAM) and the carcino-embryonic antigen (CEA) subfamily. The intercellular adhesion molecules (ICAM), vascular adhesion molecule (VCAM) and leucocyte function-associated antigens (LFA) also belong to this family.⁶

The *cadherins* are Ca²⁺-dependent CAMs, which bind cells by means of homophilic interaction. Among the better characterized cadherins are: E-cadherin (also known as L-CAM), N-cadherin and P-cadherin. Each of the cadherins displays a unique pattern of tissue distribution.⁶

The *integrin superfamily* are mediators of cell-cell and cell-extracellular matrix adhesion. Integrins are divided into subfamilies, each with a common β -subunit capable of associating with a group of α -subunits. The α - and β -subunits in various combinations form at least 20 different types of integrins. The β 1 (also known as very late activation or VLA-antigens) integrins comprise a subfamily in which eight α chains combine with one β (the β 1) chain. The vitronectin receptors share a common α v chain.⁷

The *selectins* are adhesion-receptor glycoproteins with an epidermal growth factor (EGF)-like domain. They mediate the migration of neutrophilic granulocytes in developing inflammatory reactions and are found on endothelial cells and leucocytes (ELAM).⁶

Cell surface receptors, which mediate cell-cell and cell-matrix interaction in processes like metastasis, have been the subject of intense investigation during the past decade; however, studies on uveal melanomas are few and so far limited to the Ig superfamily and the integrins. ICAM-1 and a related melanoma-associated CAM (MUC 18) have been studied in the context of metastatic behavior^{32,62} of cutaneous melanomas. Some investigators found expression correlated with metastasis,⁶² but others demonstrated that these adhesion molecules occur on a full range of benign and malignant melanocytic lesions.³² In uveal melanomas, cell adhesion molecule ICAM-1 could not be detected¹⁰ and melanoma-associated CAM AMF-7 was not expressed¹⁰⁹ or poorly expressed^{10,104} compared to cutaneous melanomas. Others found ICAM-1 on most of the uveal melanomas,^{97,104} preferentially on the mixed and epithelioid cell type.⁹⁷ It has been demonstrated that NCAM isoforms, which lack the HNK-1 epitope, might play a role in the organ specific metastatic behavior of uveal melanomas.⁹⁴ For cutaneous melanomas it has been suggested that loss of VCAM-1 may be important in the development of metastases.³²

The VLA-2 (integrin $\alpha 2 \beta 1$)⁶⁸ and the vitronectin receptor ($\alpha \nu \beta 3$)^{3,28} have been shown to be preferentially expressed in vertical growth phase of primary cutaneous melanoma lesions and metastases, suggesting a role in melanoma progression. In contrast to cutaneous melanomas, expression of the $\alpha 2$ integrin was rare in uveal melanomas, and $\alpha 5$ expression was found in all lesions.^{4,79} Furthermore, $\alpha \nu \beta 3$ was not detected in any of the primary uveal melanomas, but in two out of four metastases.⁴ All primary lesions strongly expressed $\alpha \nu \beta 5$.^{4,79} In contrast to cutaneous melanoma, it seems that determination of the integrin expression profile is not suitable for categorizing uveal melanomas as less malignant or highly malignant lesions.

CELL-MATRIX INTERACTION

During several steps of tumor development, proteolytic degradation of the extracellular matrix and other tissue barriers is required.⁹⁰ Tumor- or host-derived proteinases are major participants in this process. Different proteolytic enzyme systems are involved, including the matrix metalloprotease system²⁰ and the plasminogen activator-plasmin sys-

tem.²⁹ For cutaneous melanomas, it has been demonstrated that expression of plasminogen activators (urokinase- and tissue type plasminogen activators: u-PA and t-PA), their inhibitors, and urokinase receptor emerges in late stages of tumor progression.¹³⁴ Little is known about the role of proteases in the progression of uveal melanomas. An involvement of proteases in metastatic spread of uveal melanoma has recently been suggested by Cottam et al,²³ who detected the 72 and 92 kD type IV collagenase in culture medium of 15 primary cultures of uveal melanomas. Furthermore, t-PA activity in supernatants of primary cultures of uveal melanoma seemed to correlate with scleral invasion in the tumor lesion, whereas no u-PA activity could be detected.²¹ A histochemical study of uveal melanomas revealed that t-PA is markedly present at the invasive front, but no relation with tumor related death could be established. Expression of u-PA correlated with occurrence of metastases. The involvement of the PA system in uveal melanomas differed from that in cutaneous melanomas.¹³³ It is conceivable that in uveal melanomas other proteolytic systems are involved in metastatic spread.

Several cytokines, secreted either by tumor cells, stromal cells or TILs, may act to enhance the invasive potential of tumor cells, leading to metastasis.²⁰ The most important cytokines are: tumor necrosis factor α (TNF α), interleukin-1 (IL-1), transforming growth factor α and β (TGF α/β) and epidermal growth factor (EGF). Cutaneous melanoma cell lines, when treated in vitro with TNF α ⁷³ or IL-1 display enhanced metastasis upon injection in nude mice. Studies on uveal and cutaneous melanoma cell lines have shown that TGF β , used as a co-stimulant with TNF α or IL-1, selectively augments expression of the 92-kD type IV collagenase.²² Detectable levels of TNF α have been found in one out of 16 uveal melanomas, occurring in one out of two patients who developed metastatic disease.³⁹

The role of cell-cell and cell-matrix interactions, major elements in the acquisition of metastatic capacity of uveal melanoma, needs to be further elucidated.

Discussion

Tumors of the choroid and ciliary body pose a serious threat to life. Management of primary uveal melanoma is still controversial.^{118,119} It is still generally agreed that enucleation is necessary for many large choroidal and ciliary body melanomas. It seems justified to treat clinically small, "active" melanomas with some form of treatment.¹²¹ Most medium-sized melanomas and many large melanomas are being treated today by irradiation with or without thermotherapy, or by enucleation.¹¹⁹ It

has been suggested that modifications of local treatment will not result in any significant improvement in survival, and that research must be directed towards treatment of metastatic disease.⁸² It is therefore important to find reasonable prognostic parameters in order to assign patients to different (future) treatment modalities. These prognostic parameters just help physicians to counsel patients, which is important for the patients' quality of life. It is therefore necessary to increase our knowledge of the mechanisms underlying metastases and the identification of reliable progression parameters as prognostic parameters in primary uveal melanoma.

In cases of irradiation no tissue will be available for histopathological evaluation. For irradiated melanomas, a combination of clinical prognostic parameters with a high sensitivity and high specificity needs to be evaluated in order to select patients at high risk of metastatic disease. Histologically, LTD appears as one of the most significant and reproducible parameters in predicting survival. However, the sensitivity of LTD as a prognostic parameter has reported to be 76%, the specificity was only 44%.¹⁹ A suitable covariate might be clinical determination of the vascular pattern of the tumor. Vascular patterns in a histological slide can be visualized in a simple way in daily routine. The presence of closed vascular loops has recently been proclaimed to be the parameter most significantly associated with tumor related death of all variables tested.^{42,43,103,113} This may have a clinical diagnostic value, including the recognition of closed vascular patterns by the development of noninvasive techniques, such as ultrasound tissue characterization,¹⁵ in order to grade the biologic potential prior to treatment.

Techniques of intraocular biopsy, particularly transvitreal fine needle aspiration biopsy (FNAB) using a 22-gauge needle, have been employed in selected instances where the diagnosis cannot be established with less invasive methods. This technique has proven to be a safe and useful technique.^{12,120} In clinical atypical cases FNAB can be reliable in differentiating amelanotic choroidal melanoma from choroidal metastasis and other amelanotic fundus lesions.¹²⁰ Techniques such as DNA-ploidy measurements and immunohistochemical determination (i.e., proliferative activity: Mib-1 and c-myc) can be applied to FNAB specimens from intraocular tumors. Tentatively, these methods might also be applied on FNAB specimens from uveal melanomas which will be treated by irradiation, in order to grade the biologic potential of the tumor prior to treatment: a clinically small melanoma with spindle cells, a DNA diploid

pattern and low proliferative activity is associated with a rather favorable prognosis.^{11,17,66,93,95}

It is apparent that important trends have emerged from retrospective prognostic studies of enucleated melanomas. There is a need for a prospective study on a large series of enucleated uveal melanomas to investigate the prognostic value of histopathological covariates like LTD, the vascular pattern, cell type, the Mib-1 index, c-myc oncoprotein, TIL, melanoma-associated antigens, DNA-ploidy, morphometric analysis, and cytogenetic analysis. Ideally, histopathological prognostic parameters should be simple to assess, reproducible, inexpensive and amenable to analysis in conventionally processed tissue specimens. Cytogenetic analysis, DNA-ploidy studies and morphometric analysis are time-consuming and costly methods, and require an elaborate specially designed system. In contrast, the vascular pattern, LTD, cell type, TIL, and immunohistochemical parameters (Mib-1, c-myc, melanoma-associated antigens) can routinely be determined in an ophthalmic pathology laboratory and are relatively inexpensive. With the results of a large prospective study, a combination of clinical and histopathological prognostic parameters might be selected with high sensitivity and specificity, which may provide a way of selecting patients at high risk of developing metastatic disease. If an effective systemic treatment for metastatic uveal melanoma becomes available, early administration as an adjuvant to primary treatment may provide the best strategy for control of systemic spread.

Since it is unclear whether uveal and cutaneous melanoma respond in the same manner to adjuvant treatment, it is important to compare uveal and cutaneous melanoma with respect to the gaining of metastatic potential, i.e., tumor progression. Organ-specific colonization (i.e., liver metastasis from uveal melanomas) often follows very specific interactions between the cancer cell and the target organ, either in terms of specific cellular adhesion or growth promotion.^{38,98,115} Differences between uveal and cutaneous melanomas have been found with regard to cell-cell interaction and the proteinase systems, involved in the degradation of extracellular matrix. One explanation for these findings may be the stromal origin of the uveal melanocytes, from which uveal melanomas develop. In the early stages of virtually every malignant melanoma of the skin, atypical melanocytes are arranged at the dermoepidermal junction, which then progress through all levels of the epidermis and eventually break through the basement membrane into the dermis. A pre-invasive, intra-epithelial precursor lesion is not recognized in uveal melanoma.

This may explain the differences in components involved in cell-cell and cell-matrix interaction.

Patients with malignant melanoma may develop a cellular and/or humoral immune response to melanoma-associated antigens expressed by the tumor cells. The specific surface phenotype of melanoma-associated antigens differs markedly between uveal and cutaneous melanoma. This might have implications for the choice of a possible adjuvant therapy.

The molecular differences between metastasizing and nonmetastasizing cells are not well known. The products of oncogenes also play a role in the metastatic competence of a tumor. However, it is still not known to what extent or in what way oncogenes control the acquisition of cancerous properties or if they control the manifestation of metastases.⁵ Considerable differences between cytogenetic findings in cutaneous and uveal melanomas have been demonstrated. Molecular biological findings of the metastatic potential of uveal melanoma are so far only of scientific interest, but may in the future provide tools to develop modified treatment strategies targeted to one or more steps in progression of uveal melanoma.

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