Neoplasia • Vol. 3, No. 3, 2001, pp. 245–254 245
www.nature.com/neo

Chromosome 7 Aneusomy. A Marker for Metastatic Melanoma?

Expression of the Epidermal Growth Factor Receptor Gene and Chromosome 7 Aneusomy in Nevi, Primary Malignant Melanomas and Metastases[1]

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Abstract

Receptor tyrosine kinases such as the epidermal growth factor receptor (EGFR) play an important role in a variety of malignant neoplasias, making the search for aberrations in the relevant chromosomes an important issue. Differential expression of the *EGFR* gene was investigated by reverse transcriptase (RT)-PCR on tissue samples of normal skin, nevi, primary melanomas, and melanoma metastases. The *EGFR* gene is located on chromosome 7p12.3-p12.1. To determine the number of chromosomes 7 in cell nuclei of the mentioned tissue samples we performed fluorescence *in situ* hybridization (FISH) on touch preparations, using a DNA probe that hybridizes specifically to the centromeric region of chromosome 7. Additionally, chromosome 7 number in interphase nuclei was determined in short-term primary cell cultures of nevi, primary melanomas, and metastases. The highest *EGFR* gene expression frequency was found in melanoma metastases. By FISH we detected the highest fraction of cell nuclei with more than two chromosomes 7 in the group of metastases. Our results suggest that overexpression of the *EGFR* gene might play an important role in metastasis of malignant melanoma. This is well reflected by polysomy 7, possibly accounting for an increased *EGFR* gene copy number. *Neoplasia* (2001) **3,** 245–254.

Keywords: malignant melanoma, EGFR, chromosome 7, FISH, metastases.

Introduction

Despite the rising incidence rates and the potentially lethal outcome of malignant melanoma, little is known on the pathogenetic mechanisms causing this cutaneous neoplasia. Of special interest are the supposedly consecutive steps from a benign melanocytic nevus to the malignant melanoma and the formation of metastases. As has been shown for numerous malignancies, this malignant transformation is often associated with structural or numerical cytogenetic aberrations [1]. In human malignant melanoma, numerical and structural abnormalities of chromosomes 1, 3, 6, 9, 10, 11, and 17 were the most frequently described [2–13].

In recent years a plethora of proto-oncogenes, oncogenes, and tumor suppressor genes have been investigated in malignant melanoma to identify causative genetic alterations. Among these, receptor tyrosine kinases (RTKs) have emerged as a highly interesting group of cell membrane–bound receptors involved in signal transduction processes. There is compelling evidence supporting the involvement of RTKs in human carcinogenesis [14–19]. RTKs are regulatory transmembrane proteins that transmit biological signals from the extracellular environment to the interior cell departments. This signal transduction system has been implicated to regulate cellular functions such as cell proliferation or differentiation [20]. Epidermal growth factor receptor (EGFR), first studied in the epidermoid carcinoma cell line A431 [21], was shown to contribute to or cause malignant cell transformation in gastrointestinal, urinary, and reproductive tract malignancies, brain tumors, lung carcinoma [22], cutaneous squamous cell carcinomas, and melanomas [23]. Although mutations of RTKs as pathogenetic factors for malignant transformation, such as the mutated EGFR in human gliomas [24,25], have been reported, the prevalent RTK-related defect appears to be abnormal overexpression caused by amplification or impaired control of gene expression.

Due to controversial results and the lack of expression studies on the RNA level in melanoma and nevus tissue, we performed comparative EGFR expression analysis in normal skin, nevi, primary melanoma, and melanoma metastases. The *EGFR* gene is located on chromosome 7p12.3-p12.1.

Abbreviations: ALM, acrolentiginous malignant melanoma; EGFR, epidermal growth factor receptor; FISH, fluorescence *in situ* hybridization; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LMM, lentigo malignant melanoma; Mel.Met., melanoma metastasis; MM, malignant melanoma (unclassified); NMM, nodular malignant melanoma; NCN, nevus cell nevus; RT, reverse transcriptase; RTK, receptor tyrosine kinase; SSM, superficial spreading melanoma; UT, unaffected tissue

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[1]This work was partly supported by a grant of the Bundesministerium für Bildung, Forschung und Technologie (07 UV B 56/0).
Received 30 October 2000; Accepted 15 January 2001.

As altered gene expression can be due to gene dosage effects resulting from either more or less than the usual two gene copies per nucleus, we examined the abovementioned tissue types for the copy number of chromosome 7 by fluorescence *in situ* hybridization (FISH). FISH enables the detection of numerical and structural chromosomal aberrations in both interphase cell nuclei and metaphase spreads. For enumeration of chromosome copy numbers, chromosome specific centromeric DNA probes can be used. As FISH on thin tissue sections faces the problem that signals could be missed due to only one single section plane, we performed FISH on cell nuclei derived from touch preparations of fresh tumor tissue and on nuclei derived from short-term primary cell cultures.

Material and Methods

Clinical Material

Fresh tissue samples were obtained from patients, frozen in liquid nitrogen immediately after excision, and stored at −80°C until preparation for interphase FISH or RNA. For FISH we obtained tissues from 8 melanocytic nevi, 18 primary cutaneous malignant melanomas [including superficial spreading melanomas (SSM, n=7), lentigo malignant melanomas (LMM, n=2), nodular malignant melanomas (NMM, n=5), and unclassified melanomas (MM, n=4)], and 41 cutaneous and lymph node metastases. Seven control samples were obtained from normal, non-neoplastic tissue and safety margins. All diagnoses had been confirmed histologically.

Additionally, we established short-term primary cell cultures of nevi, primary melanomas, and melanoma metastases. We examined primary cultures from two nevi, one primary malignant melanoma, and three melanoma metastases by interphase FISH.

For reverse transcriptase (RT)-PCR analysis of *EGFR* gene expression we investigated tissue specimens taken from normal skin (n=16), nevi (n=28), primary malignant melanomas (n=47), and melanoma metastases (n=14). Eight of the 16 samples from normal skin had been excised from non–UV-exposed gluteal skin to avoid alterations of gene expression levels by ultraviolet radiation. Primary melanomas consisted of 19 NMMs, 22 SSMs, 2 LMMs, 2 acrolentiginous melanomas (ALM) and 2 melanomas on nevus. Additionally, 2 locoregional cutaneous melanoma metastases and 12 distant cutaneous and lymph node metastases were studied. Tumor thickness of primary melanomas ranged from 0.2 to 12.1 mm. Mean tumor thickness for SSM was 0.86 mm, for NMM 3.17 mm. 30 melanoma patients were female, 33 were male Caucasians.

Fluorescence in Situ Hybridization (FISH)

Primary cell cultures sample preparation Fresh cutaneous tissue was minced finely, pieces were resuspended several times to separate single cells from tissue pieces and suspension was seeded in cell culture flasks. Culture media were DMEM (Gibco, Karlsruhe, Germany) containing 10% FCS (Biochrom, Berlin, Germany) or Ham's F12 (Gibco) modified with 17% FCS (Biochrom), 3% horse serum (Biochrom), Choleratoxin (83.2 ng/ml; Sigma, Deisenhofen, Germany), and PMA (10 ng/ml; Boehringer Ingelheim, Heidelberg, Germany). Cells were cultured at 37°C and 4% or 8.5% CO_2 using Ham's F12 or DMEM, respectively. At time of harvesting cells were trypsinized, incubated in hypotonic KCl (75 mM), fixed in Carnoy's solution (3:1 methanol:acetic acid), and dropped on methanol-cleaned slides. *In situ* hybridization was performed as described elsewhere [26]. For a brief description cf. Fluorescence *In Situ* Hybridization section.

Touch preparations We performed touch preparations of tissue specimens by gently touching the not fully thawed sample to the surface of a positive loaded slide. The slides were air-dried for 5 to 7 hours before fixing in 4% paraformaldehyde/1×PBS for 20 minutes. Slides were washed in 3×PBS and 1×PBS (twice) 5 minutes each, dehydrated by incubation in ethanol (30%, 60%, 80%, 95%, 100%) and air-dried. After incubation of the slides on a heating block at 55°C overnight, RNase digestion (100 μg/ml) was performed for 1 hour at 37°C in a humid chamber. Afterwards, touch preparations were incubated in 2×SSC at 75°C for 15 minutes. The slides were digested in pepsin solution (4 mg/ml in 0.9% NaCl, pH 1.5) for 15 minutes at 37°C. This reaction was stopped by washing with 2×SSC for 5 minutes. Slides were then air-dried.

Fluorescence in situ hybridization The method of interphase FISH was performed as described elsewhere [26]. In brief, we used a directly fluorescent-labeled, α-satellite DNA probe (labeled with spectrum orange; VYSIS, Downers Grove, IL) that hybridizes to the centromere region of human chromosome 7. The probe was mixed according to the manufacturer's description and applied on the slide preparations. Probe and target DNA were denatured simultaneously in a 78°C oven for 4 minutes and then hybridized at 42°C overnight in a humid chamber. Posthybridization washes were performed in 1.5 M urea/0.1×SSC at 45°C for 30 minutes and in 2×SSC at room temperature for 2 minutes. Finally, nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole) in antifade compound (containing *p*-phenylenediamine).

Analysis of interphase FISH Fluorescence signals in 100 interphase nuclei were scored per sample using a fluorescence microscope (Zeiss, Germany) equipped with light filters for different wavelengths and a digital camera connected to a computer with MacProbe software (PSI, England) for analysis. Counting of signals was performed applying the following criteria: (a) fluorescent signals were scored as true hybridization events only if they were approximately the same size and intensity as those in adjacent cells, (b) paired signals were scored as single

Table 1. FISH Signals per Nucleus for Chromosome 7 in Samples of Different Tissue Types.

Diagnosis	Case no.	Signals per nucleus (%)									
		0	1	2	3	4	5	6	7	8	>5
Controls											
Muscle	249	3.0	9.0	81.0	4.0	1.0	0.0	ND			2.0
Normal skin	235	0.0	4.0	91.0	4.0	1.0	0.0	0.0			0.0
Mucous membrane	233	3.8	6.7	83.7	4.8	1.0	0.0	0.0			0.0
Unaffected tissue	305	3.0	13.0	81.0	3.0	0.0	0.0	0.0			0.0
Unaffected tissue	312	0.0	13.0	83.0	4.0	0.0	0.0	0.0			0.0
Safety margin	310	1.0	7.0	89.0	2.0	1.0	0.0	0.0			0.0
Safety margin	480	2.0	23.0	73.0	2.0	0.0	0.0	0.0			0.0
Mean value		**1.8**	**10.8**	**83.1**	**3.4**	**0.6**	**0.0**	**0.0**			**0.3**
Standard deviation		1.4	5.8	5.5	1.0	0.5	0.0	0.0			0.7
Nevi											
Nevus giganteus	199	0.0	10.8	82.4	6.9	0.0	0.0	0.0			0.0
NCN	302	4.0	19.0	75.0	2.0	0.0	0.0	0.0			0.0
NCN	303	1.0	12.9	81.2	3.0	2.0	0.0	0.0			0.0
NCN	304	1.0	12.0	77.0	7.0	2.0	0.0	1.0			1.0
NCN	306	0.0	7.0	87.0	3.0	3.0	0.0	0.0			0.0
NCN	309	1.0	7.0	91.0	1.0	0.0	0.0	0.0			0.0
NCN	366	3.0	13.0	80.0	4.0	0.0	0.0	0.0			0.0
NCN	367	3.0	9.0	85.0	3.0	0.0	0.0	0.0			0.0
Mean value		**1.6**	**11.3**	**82.3**	**3.7**	**0.9**	**0.0**	**0.1**			**0.1**
Standard deviation		1.4	3.7	4.9	2.0	1.2	0.0	0.3			0.3
Primary melanomas											
LMM	289	0.0	11.8	82.4	4.9	1.0	0.0	0.0			0.0
LMM	295	1.0	2.0	25.0	13.0	22.0	19.0	17.0		1.0	18.0
MM	210	0.0	8.8	49.0	32.4	6.9	ND	ND			2.9
MM	268	2.0	6.0	77.0	6.0	8.0	0.0	1.0			1.0
MM	308	2.0	4.0	91.0	2.0	1.0	0.0	0.0			0.0
MM	363	0.0	1.0	77.0	12.0	5.0	4.0	1.0			1.0
NMM	209	0.0	16.4	76.2	6.6	0.8	0.0	0.0			0.0
NMM	267	0.0	4.0	70.0	10.0	1.0	6.0	9.0			9.0
NMM	290	1.0	17.0	79.0	2.0	1.0	0.0	0.0			0.0
NMM	291	0.9	16.8	76.6	5.6	0.0	0.0	0.0			0.0
NMM	293	2.0	5.0	84.0	7.0	1.0	1.0	0.0			0.0
SSM	211	0.0	7.7	81.7	7.7	1.9	0.0	0.0			1.0
SSM	236	2.0	13.0	70.0	8.0	4.0	0.0	0.0			3.0
SSM	250	0.0	5.0	83.0	7.0	5.0	0.0	0.0			0.0
SSM	307	2.0	10.0	78.0	6.0	4.0	0.0	0.0			0.0
SSM	315	1.0	5.0	89.0	4.0	1.0	0.0	0.0			0.0
SSM	352	1.0	11.0	80.0	6.0	1.0	1.0	0.0			0.0
SSM	474	1.0	10.0	81.0	8.0	0.0	0.0	0.0			0.0
Mean value		**0.9**	**8.6**	**75.0**	**8.2**	**3.6**	**1.8**	**1.6**			**2.0**
Standard deviation		0.8	4.9	14.9	6.5	5.0	4.6	4.4			4.4
Metastases											
Mel.Met.	195	0.0	8.0	62.0	27.0	3.0	0.0	0.0			0.0
Mel.Met.	205	0.0	10.9	76.2	12.9	0.0	0.0	0.0			0.0
Mel.Met.	206	0.0	18.8	75.0	3.8	2.5	0.0	0.0			0.0
Mel.Met.	208	0.0	13.9	72.3	8.9	5.0	0.0	0.0			0.0
Mel.Met.	218	2.0	8.0	80.0	8.0	2.0	0.0	0.0			0.0
Mel.Met.	234	0.0	4.7	68.0	26.0	0.7	0.0	0.0			0.7
Mel.Met.	265	1.0	0.0	33.0	14.0	21.0	27.0	4.0			4.0
Mel.Met.	266	0.0	1.0	15.0	7.0	28.0	44.0	5.0			5.0
Mel.Met.	292	1.0	2.0	16.0	18.0	21.0	19.0	21.0	2.0		23.0
Mel.Met.	294	1.0	7.0	88.0	2.0	1.0	1.0	0.0			0.0
Mel.Met.	311	0.0	4.0	39.0	36.0	20.0	1.0	0.0			0.0
Mel.Met.	313	0.0	8.0	63.0	15.0	12.0	2.0	0.0			0.0

(continued on next page)

Table 1. (*continued*).

Diagnosis	Case no.	Signals per nucleus (%)									
		0	1	2	3	4	5	6	7	8	>5
Mel.Met.	314	0.0	7.0	51.0	34.0	7.0	1.0	0.0			0.0
Mel.Met.	337	0.0	11.0	55.0	34.0	0.0	0.0	0.0			0.0
Mel.Met.	338	0.0	2.0	17.0	8.0	25.0	35.0	12.0	1.0		13.0
Mel.Met.	339	0.0	7.0	47.0	14.0	30.0	1.0	0.0	1.0		1.0
Mel.Met.	340	0.0	2.0	21.0	73.0	3.0	0.0	1.0			1.0
Mel.Met.	348	2.0	14.8	72.1	9.8	0.0	0.0	0.0			0.0
Mel.Met.	349	0.0	13.8	48.3	24.1	13.8	0.0	0.0			0.0
Mel.Met.	350	1.0	8.9	54.5	25.7	8.9	1.0	0.0			0.0
Mel.Met.	351	1.0	13.9	53.5	10.9	5.0	9.9	5.0	1.0		6.0
Mel.Met.	355	0.0	1.0	49.0	48.0	2.0	0.0	0.0			0.0
Mel.Met.	364	1.0	5.0	66.0	17.0	3.0	2.0	6.0			6.0
Mel.Met.	365	1.0	10.0	46.0	39.0	1.0	1.0	2.0			2.0
Mel.Met.	369	0.0	5.0	84.0	5.0	3.0	3.0	0.0			0.0
Mel.Met.	370	1.0	3.0	27.0	69.0	0.0	0.0	0.0			0.0
Mel.Met.	415	0.0	5.0	54.0	39.0	2.0	0.0	0.0			0.0
Mel.Met.	416	0.0	0.0	16.0	7.0	11.0	40.0	22.0			26.0
Mel.Met.	417	0.0	0.9	34.0	53.8	9.4	1.9	0.0			0.0
Mel.Met.	418	1.0	13.0	82.0	2.0	2.0	0.0	0.0			0.0
Mel.Met.	420	0.0	1.0	9.0	47.0	40.0	2.0	0.0	1.0		1.0
Mel.Met.	421	1.0	7.0	40.0	49.0	3.0	0.0	0.0			0.0
Mel.Met.	422	0.0	2.0	29.0	52.0	13.0	1.0	2.0		1.0	3.0
Mel.Met.	423	1.0	8.0	74.0	5.0	2.0	5.0	3.0	2.0		5.0
Mel.Met.	425	0.0	4.0	22.0	7.0	16.0	37.0	11.0	3.0		14.0
Mel.Met.	470	0.0	4.4	75.0	15.2	2.2	2.2	1.1			1.1
Mel.Met.	471	1.0	8.0	88.0	3.0	0.0	0.0	0.0			0.0
Mel.Met.	473	0.0	2.9	57.4	35.3	1.5	1.5	1.5			1.5
Mel.Met.	475	0.0	2.0	11.0	86.0	1.0	0.0	0.0			0.0
Mel.Met.	477	0.0	5.0	84.0	8.0	3.0	0.0	0.0			0.0
Mel.Met.	481	0.0	8.0	50.0	37.0	4.0	0.0	1.0			1.0
Mean value		**0.4**	**6.4**	**51.3**	**25.3**	**8.0**	**5.8**	**2.4**			**2.8**
Standard deviation		0.6	4.6	23.6	21.0	9.7	12.1	5.1			5.9

NCN, nevus cell nevus. *LMM*, lentigo malignant melanoma. *NMM*, nodular malignant melanoma. *SSM*, superficial spreading melanoma. *MM*, malignant melanoma (unclassified). *Mel.Met.*, melanoma metastasis. ND: not determined.

events, and (c) only signals in nonoverlapping, apparently intact nuclei were scored.

Statistical analysis Statistical analysis comparing the distributions of the mean fraction of nuclei with a certain number of FISH signals in primary melanomas and melanoma metastases was performed using the two-sided Kolmogorov-Smirnov exact test. The differences were considered to be statistically significant if the *P* value was .025 or less.

RT-PCR Analysis of EGFR Gene Expression

RNA isolation A total of 105 tissue samples from patients with melanocytic nevi, cutaneous malignant melanomas, metastases, and normal skin were investigated. At time of preparation the tissue was minced and total mRNAs were isolated from homogenates using the RNA-Clean System (Angewandte Gentechnologie Systeme, Germany).

Reverse transcription Using oligo (dt) primers the extracted mRNA was reverse transcribed with the Reverse Transcriptase System (Promega, Madison, WI). The obtained cDNAs were then phenol/chloroform extracted, precipitated by ethanol extraction and redissolved in double-distilled H_2O. The cDNA concentration was measured by light absorbance at 260 nm.

PCR In PCR reactions (25 μl) 100 ng cDNA template was used to analyze expression of the *EGFR* gene. Besides, the PCR mixtures contained 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Promega), 0.2 μl Taq polymerase (5 U/μl, Boehringer Mannheim, Germany) and 1 μM sense and antisense primers: 5′-ACT AGC CAG GAA GTA CTT CC-3′ and 5′-GGC CTT CTT GGA TCT TTA GT-3′. Cycling parameters: an initial heating step (94°C, 4 minutes), followed by 93°C, 35 seconds; 60°C, 35 seconds; 72°C, 35 seconds; 35 cycles, and one final extension step (68°C, 10 minutes); predicted product size: 398 bp.

It was tested that PCR was still in the exponential phase at the end of cycling. To circumvent false-negative results we used primers detecting expression of the housekeeping gene *GAPDH* (Glyceraldehyde-3-phosphate Dehydrogenase). Genomic contamination was ruled out by the use of a

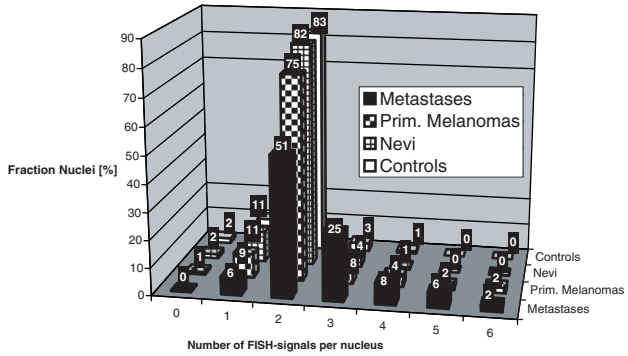


Figure 1. *Mean fraction of nuclei (%) with a certain number of FISH signals for chromosome 7 in interphase nuclei of benign and malignant tissue samples.*

further intron-spanning set of primers with different product sizes for genomic DNA and cDNA. As a positive control cDNA prepared from the melanoma cell line C32 (CRL-1585, ATCC, Rockville, MD) was amplified. Water instead of cDNA template was applied as a negative control. In parallel, 100 ng of genomic DNA was amplified. The PCR-band intensity of genomic DNA was determined as a comparison level indicating weak (sample PCR band intensity weaker than genomic DNA band) or strong (sample PCR band intensity similar or stronger than genomic DNA band) *EGFR* expression in the samples.

Following PCR, identical aliquots (5 μl) of reaction products were electrophoretically separated on 2% agarose gels and PCR products were visualized by ethidium bromide staining. Densitometric analysis of the bands was performed using PCR-analysis software (Image Master VDS; Pharmacia, Freiburg, Germany).

Results

Results of Interphase FISH for Chromosome 7

Eight melanocytic nevi, 18 primary melanomas, 41 melanoma metastases and seven control samples were analyzed for their chromosome 7 karyotype by interphase FISH using a DNA probe specific for centromere of chromosome 7. Aside from nuclei with normal chromosome 7 karyotype we observed different fractions of nuclei with trisomy 7 as well as nuclei with monosomy 7 and such with four or more signals for chromosome 7 in all examined melanoma specimen (Table 1). The number of hybridization spots per nucleus ranged from 0 to 8. The average fraction of nuclei with normal karyotypic status for chromosome 7 (two fluorescent signals) was 83.1% in control samples and 82.3% in benign nevi, respectively. In primary melanomas only 75.0% of nuclei were normal declining to melanoma metastases, where only an average of 51.3% of cell nuclei showed normal chromosome 7 karyotype (Figure 1). Nuclei with monosomy 7 were observed in approximately equal amounts in controls and nevi (mean value 10.8% and 11.3%, respectively), less in primary melanomas (8.6%), and least in metastases (6.4%). However nuclei with trisomy 7 were found much more frequently in metastases (mean value 25.3%) than in primary melanomas (8.2%), nevi (3.7%), and control samples (3.4%). The distributions of the mean fraction of nuclei with a certain number of FISH signals in primary melanomas and melanoma metastases (Figure 1) are significantly different (P=.0001, Kolmogorov-Smirnov exact test).

There was no correlation of FISH results with the standard pathologic prognostic factors Breslow thickness, Clark level,

Table 2. Clinical Data of Primary Malignant Melanomas and Chromosome 7 FISH Results, Sorted on Clark Level.

Diagnosis	Case no.	Localization	Breslow thickness (mm)	Clark level	Metastasis	Signals per nucleus (%)									
						0	1	2	3	4	5	6	7	8	>5
SSM	474	hand	<0,75	I	neg	1.0	10.0	81.0	8.0	0.0	0.0	0.0			0.0
LMM	289	head	0.3	II	ND	0.0	11.8	82.4	4.9	1.0	0.0	0.0			0.0
SSM	236	thigh	1.04	II	neg	2.0	13.0	70.0	8.0	4.0	0.0	0.0			3.0
SSM	352	back	1.04	II	neg	1.0	11.0	80.0	6.0	1.0	1.0	0.0			0.0
MM	268	back	0.7	III	ND	2.0	6.0	77.0	6.0	8.0	0.0	1.0			1.0
NMM	209	shoulder	2.1	III	ND	0.0	16.4	76.2	6.6	0.8	0.0	0.0			0.0
SSM	315	back	0.38	III	ND	1.0	5.0	89.0	4.0	1.0	0.0	0.0			0.0
MM	363	abdomen	1.69	IV	pos	0.0	1.0	77.0	12.0	5.0	4.0	1.0			1.0
NMM	267	back	11	IV	ND	0.0	4.0	70.0	10.0	1.0	6.0	9.0			9.0
NMM	291	lower leg	5.25	IV	pos	0.9	16.8	76.6	5.6	0.0	0.0	0.0			0.0
NMM	293	lower leg	5.25	IV	pos	2.0	5.0	84.0	7.0	1.0	1.0	0.0			0.0
SSM	250	ear	5	IV	pos	0.0	5.0	83.0	7.0	5.0	0.0	0.0			0.0
LMM	295	ND	ND	ND	ND	1.0	2.0	25.0	13.0	22.0	19.0	17.0		1.0	18.0
MM	210	ND	ND	ND	ND	0.0	8.8	49.0	32.4	6.9	ND	ND			2.9
MM	308	ND	ND	ND	ND	2.0	4.0	91.0	2.0	1.0	0.0	0.0			0.0
NMM	290	ND	ND	ND	ND	1.0	17.0	79.0	2.0	1.0	0.0	0.0			0.0
SSM	211	back	0.8	ND	ND	0.0	7.7	81.7	7.7	1.9	0.0	0.0			1.0
SSM	307	ND	ND	ND	ND	2.0	10.0	78.0	6.0	4.0	0.0	0.0			0.0
Mean value						0.9	8.6	75.0	8.2	3.6	1.8	1.6			2.0
Standard deviation						0.8	4.9	14.9	6.5	5.0	4.6	4.4			4.4

neg, negative. *pos*, positive. ND: not determined.

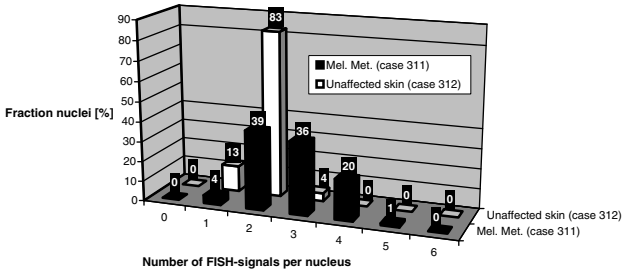


Figure 2. *Fraction of nuclei (%) with a certain number of chromosomes 7 in samples of melanoma metastasis and adjacent unaffected skin from the same patient.*

and metastasis in cases of primary melanomas where clinical data was available (Table 2).

Figure 2 illustrates a case where a melanoma metastasis (case 311) and the clinically unaffected skin adjacent to the metastasis (case 312) from the same patient were available for FISH investigation. The fraction of cell nuclei with normal chromosome 7 karyotype increased from 39% in the metastasis to 83% in the adjacent skin, whereas the fraction of nuclei with trisomy 7 decreased from 36% to 4%. In this case no nuclei with four chromosomes 7 were detectable in the adjacent tissue but 20% of metastatic nuclei showed this tetrasomy 7.

Based on previously published results [27] chromosome 7 number in tumor cells was regarded as an essential chromosomal component of the tumor if 20% or more of nuclei showed a certain number of copies. Of the investigated metastases, 19/41 (46.3%) showed trisomy 7 in at least 20% of nuclei, whereas only 1/18 (5.6%) primary melanomas, and none of the nevi and controls fitted this criteria.

Compared with primary melanomas (3.6%), nevi (0.9%), and controls (0.6%), cell nuclei with four chromosomes 7 appeared most frequently in metastatic cases (8.0%). Seven of 41 metastases (17.1%) showed four chromosomes 7 in at least 20% of nuclei, compared to only 1/18 (5.6%) primary melanomas and none of the nevi (n=8) and controls (n=7).

Five chromosomes 7 in more than 20% of nuclei were found in 5/41 metastases (12.2%) but in none of the 18 primary melanomas. No cell nuclei with five chromosomes 7 were scored in nevi and control samples. Two metastatic samples (cases 292 and 416) showed even more than 20% of nuclei with six signals for chromosome 7, whereas none of the primary melanomas, nevi and controls fitted this criteria.

Results differed among the individual cases. Looking at single cases of primary malignant melanomas the highest fractions of nuclei with three or four chromosomes 7 were 32.4% (case 210) and 22% (case 295), respectively. In single metastatic cases, we detected up to 86% of nuclei with trisomy 7 (case 475, Figure 3) and up to 40% of nuclei with four (case 420), 44% with five (case 266) and 22% (case 416, Figure 4) with six chromosomes 7. Metastatic case 420 even showed 90% of nuclei with more than two chromosomes 7.

To sum up, we detected nuclei with more than two hybridization signals for chromosome 7 in nevi and control cases in less than 5% (mean values), in primary melanomas

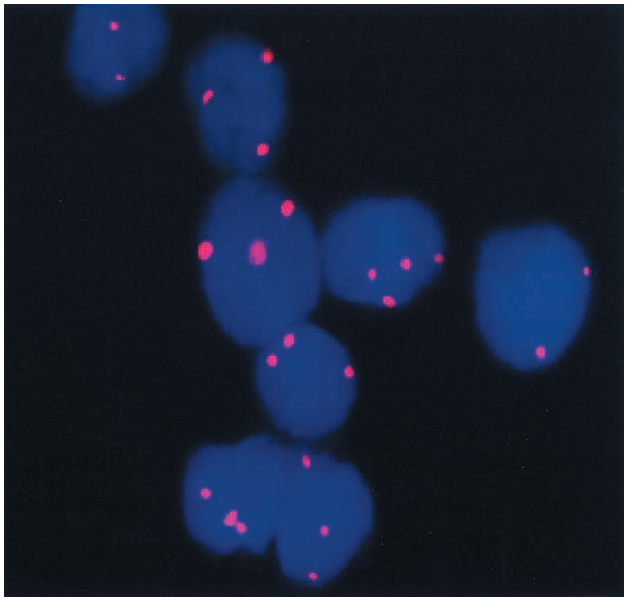


Figure 3. *Interphase nuclei of a melanoma metastasis (case 475) showing two to four FISH signals for chromosome 7.*

in about 15%, but in metastases an average of more than 41% of nuclei revealed more than two chromosomes 7 (Table 1). The observed difference between primary melanomas and metastases is statistically significant (P=.0001).

Additionally, we performed interphase FISH for chromosome 7 on nuclei derived from primary cell cultures of two nevi, one primary melanoma, and three melanoma metastases. In nuclei of the two cultured nevi, we found a similar fraction of 82% of nuclei with two chromosomes 7 and about 7% and 8% with three or one signal(s). In the cultured cells

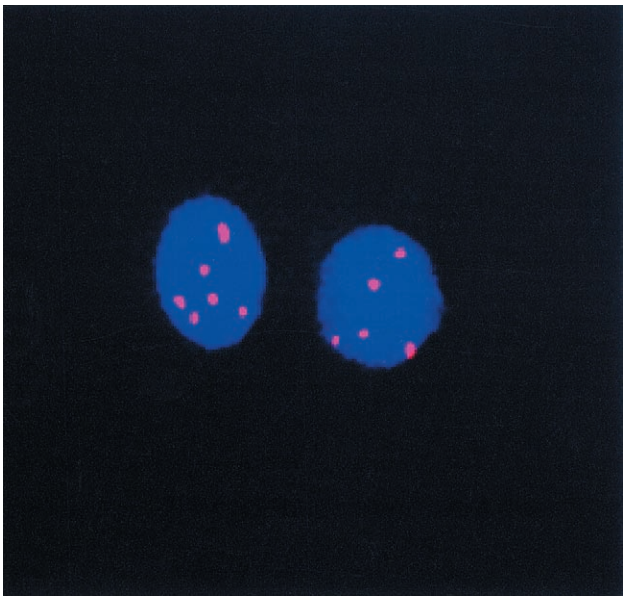


Figure 4. *Two interphase nuclei of a melanoma metastasis (case 416) with five or six FISH signals for chromosome 7.*

of the primary melanoma, 84% of nuclei showed a normal chromosome 7 karyotype and no nuclei with additional chromosome 7 copies were found. One of the three cultured melanoma metastases showed a relatively inconspicuous chromosome 7 karyotype (88% nuclei with two signals, 8% three signals), whereas the other two cultured metastatic cases owned 84% and 92% nuclei with trisomy 7, respectively.

Results of RT-PCR analysis of EGFR Gene Expression

A total of 105 tissue samples from patients with melanocytic nevi, cutaneous malignant melanomas, and normal skin samples were studied. Clinicopathologic data concerning tumor type and, in the case of primary melanomas, tumor thickness were obtained. With regard to tumor thickness of primary melanomas, nodular malignant melanomas and superficial spreading melanomas were analyzed separately, because the average tumor thickness at time of diagnosis in general is higher in nodular malignant melanoma than in superficial spreading melanoma.

Each sample was investigated for *EGFR* and GAPDH expression. Figure 5 shows a representative example of *EGFR* RT-PCR products of different investigated tissue types and the classification in weak and strong expression relative to the PCR band intensity of genomic DNA.

Twenty-seven percent (6/22) of primary superficial spreading melanomas and even fewer nodular malignant melanomas (21%, 4/19) showed mostly strong (18% and 16%, respectively) *EGFR* RNA expression (Figure 6). *EGFR*-expressing primary melanomas had a lower tumor thickness than *EGFR*-negative melanomas. Nodular malignant melanomas expressing *EGFR* had a mean tumor thickness of 1.6 mm in contrast to 3.7 mm in *EGFR*-negative tumors. Similar results were found in superficial spreading melanomas: 0.7 mm in *EGFR*-positive versus 0.9 mm in *EGFR*-negative melanomas. One of two acrolentiginous melanomas, 2/2 lentigo malignant melanomas, 1/2 melanomas on nevus and 1/2 locoregional cutaneous melanoma metastases were *EGFR* positive.

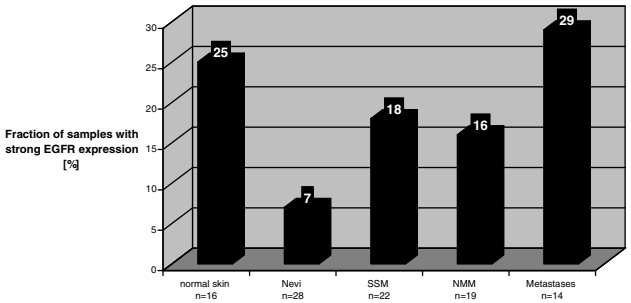
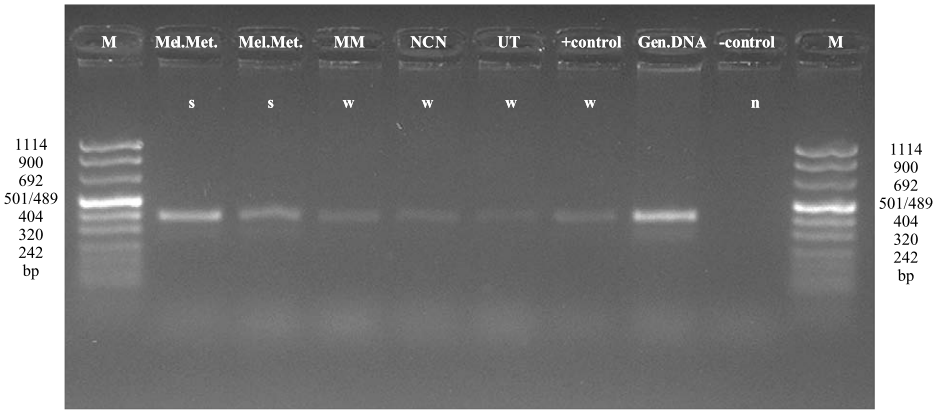


Figure 6. *EGFR gene expression frequency in different tissue types exhibiting strong expression.*

EGFR expression was undetectable in 63% of normal skin tissue (10/16). The highest *EGFR* expression frequency was found in melanoma metastases (57%, 8/14) followed by nevi (50%, 14/28). However, 43% of all examined nevi (corresponds to 86% of *EGFR*-positive nevi) expressed *EGFR* weakly, whereas only 7% (corresponds to 14% of *EGFR*-positive nevi) showed a strong *EGFR* expression level (Figure 6). This is in contrast to the findings in melanoma metastases, where 29% of all investigated cases showed strong expression (corresponds to 50% of *EGFR*-positive metastases). Thus, we found the highest frequency of strong *EGFR* expression by far in melanoma metastases, compared to all other investigated tissues.



+control: Positive control
-control: Negative control
Gen.DNA: Genomic DNA
M: Molecular weight marker
Mel.Met.: Melanoma metastasis
MM: Primary malignant melanoma
n: no expression
NCN: Nevus cell nevus
s: strong expression
UT: Unaffected tissue
w: weak expression

Figure 5. *Representative picture of agarose gel (2%) electrophoresis of EGFR RT-PCR products (product size: 398 bp).*

Discussion

The highest frequency of strong *EGFR* gene expression was detected in melanoma metastases. Overexpression of *EGFR* due to gene amplification has been reported in late-stage melanomas [28]. Our findings in metastases on RNA level are in accordance with published data obtained on protein level by immunohistochemical staining, where the highest fraction of positive stained samples was found in melanoma metastases, too [29]. Because the oncogenic effect of RTKs of subclass I derives from overexpression rather than mutation, strong expression of *EGFR* is probably the point of interest in melanoma. Thus, if we concentrate on the fraction of strong *EGFR* expression, primary melanomas lie between nevi and metastases (Figure 6). Thus, our results support the hypothesis of an increasing *EGFR* expression in human melanocytic tumor progression as postulated by de Wit et al. [29]. The relatively high rate of normal skin samples exhibiting strong *EGFR* gene expression (Figure 6) might be explained by the biological nature of skin as a tissue composed of different cell types. Whereas nevi and melanomas are thought to consist of cells with a melanocytic origin, melanocytes are only a small fraction in normal skin besides fibroblasts, keratinocytes, and other cell types. Normal human skin keratinocytes are well known to express *EGFR* [30,31], and *EGFR* expression seems to play an important role in the growth and differentiation of named cells [32]. This might probably be the reason for the relatively high *EGFR* gene expression rate observed in normal skin whereas it is still below that observed in melanoma metastases.

Using FISH, we detected the highest fraction of nuclei with chromosome 7 aneusomy in melanoma metastases. Only an average of 51% of the examined nuclei of these samples showed normal chromosome 7 karyotype whereas more than 41% exhibited more than two chromosomes 7. The detected difference between this distribution and that observed in primary melanomas is statistically significant (P=.0001). This might point to an important role of aneusomy 7 rather in the metastatic progression than in the pathogenesis of the disease.

Aneusomy 7 has often been found in other malignancies, for example in prostate cancer. Brown et al. [33] observed gain of chromosome 7 in 20% of touch preparations of prostate carcinoma samples by FISH. Barranco et al. [34] found chromosome 7 and/or chromosome 8 aneusomy in all investigated aneuploid prostate cancer samples. They found trisomy 7 to be the most frequent alteration present in 56% of aneuploid tumors. Takahashi et al. [35] detected gain of chromosome 7 in 76% of aneuploid prostate carcinomas. In primary cutaneous melanomas chromosome 7 copy number gain was found in 50% (n=32) of cases by comparative genomic hybridization [36]. The authors speculate that gain of chromosome 7 occurs late in melanoma progression. Matsuta et al. [27] detected copy number gain of chromosome 7 in 40.9% of primary and metastatic melanomas by FISH. Taking our results and the mentioned publications into account, aneusomy of chromosome 7 might play an important role in metastasis of malignant melanomas. This might be due to an increased copy number of the *EGFR* gene, which is located on chromosome 7p12.3-p12.1 and which we found most frequently expressed in melanoma metastases.

There are other genes on chromosome 7 that play a role in human malignancies and might be also involved in malignant melanoma. *B-raf* proto-oncogene is located on 7q34 [37]. The gene for platelet-derived growth factor alpha (*PDGF-A*) maps to 7p22 [38]. *PDGF-A* is expressed in primary and malignant melanoma but not in normal skin [39], and might function as an autocrine growth factor as well as an angiogenesis factor in tumor development. Plasminogen activator inhibitor type 1 (*PAI-1*) gene is located in region 7q21.3-q22 and was found to be expressed in highly invasive metastatic human melanoma cell lines but not in those lacking this characteristic [40]. This suggests a putative role of PAI-1 expression in metastasis of malignant melanoma. Another important cancer-related gene is the *MET* proto-oncogene on 7q31, encoding for a membrane receptor protein with, like EGFR, tyrosine–protein kinase activity. *MET* gene was shown to be overexpressed in sporadic papillary renal cell carcinoma where trisomy 7 is the most frequently observed cytogenetic abnormality [41], suggesting that a gene (or genes) located on chromosome 7 plays a role in the pathogenesis of this neoplasia. Wullich et al. [42] found the *EGFR* gene and the *MET* gene independently amplified in human glioma, a tumor where gains of chromosome 7 are frequent [43]. In malignant melanoma, *MET* gene was shown to be expressed at a significant level at late stages of melanoma progression (metastatic lesions) [44]. The localization of a variety of additional cancer-related genes together with *EGFR* on chromosome 7 might suggest a possible overexpression of these genes in melanoma metastases through increased gene copy numbers through the observed polysomy 7. Whether the discovered interdependence of polysomy 7 and metastasis is causative, correlative or consequential is a fascinating question from a biological viewpoint, which is going to be addressed in a consecutive study.

Our FISH results in percent are mean values and standard deviations for the mean fraction of nuclei with a certain number of FISH signals for chromosome 7 are quite high (Table 1). This is due to the partly big differences concerning chromosome 7 karyotype among the single metastatic cases. The panel ranges from metastases that appeared to have normal chromosome 7 karyotypes in almost all investigated nuclei to cases where about 90% of nuclei owned more than two chromosomes 7. There are two possibilities to explain these differences. Firstly, if we postulate that tumor cells differ in their karyotypes from normal cells, the observed differences could be due to the heterogeneity of tissue samples leading to "contamination" of touch preparations with nontumorous cells. In these cases, the fraction of nuclei with aneuploidy or aneusomy might depend on the percentage of tumor tissue in the touch preparations and might differ among the single cases. Secondly, the differences between metastases might be well founded in the possibility that there are tumors that have

a relevant fraction of cells with an aberrant chromosome 7 karyotype and others that consist mainly of inconspicuous cells, as far as chromosome 7 is concerned. Our findings in nuclei of short-term primary cultures, where identity of cells as tumor cells was clear and where we found metastases with and without numerical aberrations of chromosome 7, point to the latter hypothesis. Taking this into account, there could be a principal difference in proliferative and disseminating potential of metastases with differing chromosome 7 copy numbers. This hypothesis, though accounting a controlled prospective trial, may be the most interesting to follow.

The FISH results observed in cells derived from short-term primary cultures are comparable to those obtained from touch preparations. In future FISH investigations, short-term primary culture of sample cells will be the method of choice whenever possible, because selective effects are minimal and identity of cells is much more easy to determine. The interesting findings on the difference of aneusomy 7 rates in safety margins and melanomas or melanoma metastases might help to identify the necessary resection margin by means of this cytogenetic approach.

Gain and aberrations of chromosome 7 have been described to correlate with worse prognosis in various neoplasias. In prostate cancer gain of chromosome 7 was strongly associated with advanced tumor stages [35] and alterations of chromosome 7 were observed in 96% of patients with poor prognosis [45]. Additionally, trisomy 7 and monosomy 8 were significantly associated with poor prognosis in prostate cancer [34]. In a chromosome banding study on tumor biopsies from patients with metastatic melanoma, Trent et al. [46] observed that melanoma patients with structural abnormalities of chromosome 7 or 11 had a significantly shorter survival time than patients without these abnormalities. These observations and our findings certainly warrant further studies on the importance of increased copy numbers or aberrations of chromosome 7 as a prognostic parameter also for melanoma.

Acknowledgements

The authors thank Gerda Hack and Petra Miller for their excellent technical assistance, Dr. Oliver Zimmer for kindly supplying tissue samples, and Dr. Martina Kron and Michaela Glasbrenner (Department of Biometrics, University of Ulm) for support in statistical analysis.

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