Ph.D. THESIS

GENETIC CHARACTERIZATION OF HUMAN MELANOMAS WITH DIFFERENT BIOLOGICAL BEHAVIOR USING *IN SITU* HYBRIDIZATION METHODS

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DEBRECEN, 2004.

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INTRODUCTION

Early detection and treatment of cancers may highly improve patients' survival and recovery. Cancer research go back to more than hundred years, and although in the last few years its development has being accelerated and essential findings have been discovered, the benefit of which is already visible in the clinical practice (e.g. treatment of *erbb2*-pozitive invasive breast cancer), most of the patients with metastatic diseases may not expect complete remission. This is the case especially in malignant melanoma, the most aggressive form of skin cancers, where the five-year survival of metastatic patients is less than 50%. The aggressive behavior of melanoma is characterized by early metastatic potential, and even tumors slightly thicker than 1 mm are able to kill the patient within a short time. In the development and progression of melanoma, among alterations of genes predisposing to this malignancy, environmental factors play an essential role. Among them, the cancer initiation effect of the ultraviolet radiation is supported by the fact that in the last 10 years the incidence of malignant melanoma has increased more rapidly than any other cancers, mainly in those countries where the rate of harmful UV irradiation due to the growing thinner ozone layer has increased significantly.

The clinical, hystopatological and biological aspects of melanoma progression are well known, but our knowledge about the genetic markers related to the molecular mechanisms taking part in its development and progression and its metastatic spread is very poor. The bases of melanoma progression models were constituted of *in vitro* cell lines established exclusively from matestases, only few primary tumors have been analyzed. The best-known melanoma specific genes were discovered by studying familiar melanomas. The multistep process of tumor initiation includes alterations of many genes, however chromosomal instability, aneuploidy and genetic heterogeneity are characteristic features of melanoma from the very beginning. From

clinicopathological point of view, malignant melanoma can be subdivided into four main subtypes with different biological behavior and characterized by different genetic markers. The study of these markers and the comparison of them with clinical parameters are essential not only in the establishment of the diagnosis but also in therapy planning.

Understanding of the genetic alterations of cancer initiation and progression had been relied on the classical cytogenetic studies of chromosomal aberrations for many years, the disadvantages of which are well known. Alternative methods with high resolution, which make possible the study of chromosomal alterations without *in vitro* manipulation of the tumor genome, appeared in the early 90's. Among these, fluorescence *in situ* hybridization (FISH) and comparative genomic hybridization (CGH) have a prominent role. By FISH, nearly all chromosomal alterations (aneusomy, translocation, amplification and deletion) can be detected in interphase cells. By CGH, rapid and comprehensive analysis of the tumor genome can be carried out, series of genetic alterations can be detected in a single experiment without prior knowledge. Alterations revealed by CGH may turn the attention to those chromosomal regions which might be specific to a certain cancer type or steps of tumor progression. Detailed analyses of them may contribute to our understanding about tumor progression at the molecular level.

In my Ph.D. thesis, using conventional and the more recent FISH and CGH approaches, my aim was to study chromosomal alterations in two melanoma subtypes with different biological behaviour; determine the rate of c-*myc* oncogene amplification and compare it with clinicopathologycal parameters; perform cDNA-based array analyses of genetic changes in primary and metastatic melanoma tumor pairs; and the complex genetical analysis of a novel melanoma cell line with metastatic potential established from a superficial spreading melanoma.

AIM OF THE STUDY

The Aims of the Thesis

1. ***Interphase FISH analysis of primary melanomas with different biological behavior by chromosome centromere and c-myc gene specific DNA-probes***
   - According to our previous CGH studies, the majority of primary and metastatic melanomas had amplifications on chromosomal region 8q22-qter, where the c-*myc* oncogene is located. Therefore, our primary aim was the detailed analysis of the role of c-*myc* oncogene in superficial spreading and nodular melanoma subtypes and their corresponding metastases.
   - Analyses of the relationship between aneuploidy and numerical aberration of c-*myc* oncogene and the clinicopathological parameters of primary melanomas.

2. ***Complex genetic analysis of a novel human melanoma cell line with metastatic potential***
   - Comparison of the genetic alterations of a melanoma cell line (M35/01) and its original superficial spreading primary tumor by chromosomal CGH.
   - Detailed study of numerical and structural changes of the cell line by FISH methods with different resolution and array-CGH.

3. ***cDNA-based array analysis of primary melanomas and their metastases***
   - cDNA-based array comparison of genetic alterations in primary-metastatic tumor pairs and in a melanoma cell line.

MATERIALS AND METHODS

1. Melanoma samples

Melanoma tissues were obtained from the Department of Dermatology, University of Debrecen, Debrecen, Hungary and Department of Dermatology, National Institute of Oncology, Budapest, Hungary. The tumor samples consisted of 28 nodular, 26 superficial and 2 acral melanomas and 14 corresponding metastatic lesions (i.e. primary and metastatic tumors were removed from the same patient). The staging and the four thickness categories of primary melanomas were based on the new TNM melanoma staging system. Except one case, patients did not undergo chemotherapy or radiotherapy prior to the surgery of their primary tumors.

2. FISH analysis

DNA-probes were directly or indirectly labeled by nick translation. FISH was carried out as described earlier. Briefly: nuclei or metaphase chromosomes were denatured in hybridization solution at 75°C-on for 2.5-5 minutes, than dehydrated and air dried. Hybridization mix was denatured at 73C° for 5 minutes. Hybridization was carried out in a humidified chamber at 37°C overnight. Non-hybridized DNA-probes were removed by washes. Nuclei were counterstained with diamino-fenilindol (DAPI) or propidium-iodid in anti-fade solution.

Chromosome index: total number of signal spots/number of nuclei counted. The copy number category for monosomy reflects the major (> 50%) or dominant (20–50%) cell population present. Polysomy was defined if more than two hybridization signals were found in more than 15% of the cells counted.

After hybridization, samples were scored for the number of fluorescent signals in each nucleus by using a fluorescent microscope equipped with

selective filters for the detection of FITC, SpectrumGreen, SpectrumOrange and DAPI. Approximately 200-500 nuclei and/or 10 metaphases were scored for each specimen. Three-color images were captured using a digital imaging analysis system. In all experiments, normal lymphocytes were used as hybridization controls.

3. Spectral karyotyping

Spectral karyotyping was performed as previously described. Briefly, chromosome specific libraries generated by PCR from flow-sorted human chromosomes were directly labeled with nucleotides conjugated to five different dyes (FITC, Rhodamine, Texas Red, Cy5 and Cy5.5). All 24 chromosomes libraries were hybridized simultaneously to the metaphases. After washing, the slides were stained with DAPI in antifade medium. The discrimination between the different spectra was done using the SD300 spectral bio-imaging system. The system enables the measurement of the full visible light spectrum at each pixel of the image by using a Sagnac interferometer. A classification algorithm was used to differentiate between different spectra in the image and to assign pseudocolors to all the pixels which have similar spectral characteristics. The DAPI image was captured separately and inverted to give a G-banding pattern. The chromosomes were then sorted automatically into a karyotype table.

3. Comparative genomic hybridization

CGH was done according to a published protocol. Briefly, tumor cell DNA and normal female reference DNA were extracted using PCI extraction protocol and labeled with fluorescein–deoxyuridine triphosphate nucleotide and Texas-Red-dUTP using standard nick translation. Labeled DNAs (200 ng each) and 20 μg of unlabeled Cot-1 DNA were hybridized onto commercially

available normal metaphase chromosomes. The hybridizations were evaluated using a commercial digital image analysis system.

Evaluation of CGH hybridizations was performed by a computerized quantitative image analysis system attached to a Zeiss Axioplan fluorescence microscope. Acquisition of fluorescence images (8-10 metaphases per sample) was done by a monochrome CCD camera. After automatized background correction, chromosome karyogrames were constructed on the bases of DAPI-images. Determination of chromosomal aberrations was done on the bases of the rate of green/red fluorescence intensities. DNA gains reflects the alterations with >1.15 green/red fluorescence intensity; and DNA losses <0.85 green/red fluorescence intensity. Diagnostical background was determined from the averages of normal-normal hybridizations.

4. Array-CGH

Microarrays containing 2460 overlapping bacterial artificial chromosome (BAC) clones in triplicate, or 12300 cDNA elements were produced as described previously. In short, cDNAs or DNA isolated from BAC clones were amplified using ligation mediated PCR to generate representations of the human DNAs. The DNAs were spotted on chromium-coated slides (BAC arrays) or glass slides (cDNA arrays) using a costume-built arrayer. Scanning and high-resolution arrays were used as described elsewhere.

One μg of test and reference genomic DNA were labeled by random priming with Cy3 dUTP and fluorescein dUTP, respectively. Non-incorporated nucleotides were removed using Qiagen QIAquick PCR Purification Kit. Labeled DNA was mixed with Cot-1 and ethanol precipitated, than dissolved in hybridization mix to achieve a final of 50% formamide, 10% dextran sulphate, 2xSSC, 4% SDS, and 10μg/μl of yeast tRNS. The hybridization solution was heated to 72ºC for 10 minutes, than

incubated at 37ºC for 1 hour to allow blocking of the repetitive sequences. A wall enclosing the array was made with rubber cement and the resulting well was filled with hybridization mix. Slides were placed at 37ºC for 48 hours. After hybridization, slides were washed once in 50% formamide, 2xSSC, pH 7 45ºC-on for approximately 15 minutes, once in 2xSSC/0.1%SDS at 45ºC for 20 minutes, and in 0.1 mol/L sodium-phosphate and 0.1% Nonidet P-40 at room temperature for 10 minutes. After air-dried, array was mounted in antifade solution containing DAPI (1 μg/ml) to counterstain the DNA targets.

Image acquisition and analysis, and data extraction were performed as described previously. Spots with >0.6 Log2 value were considered as gains and values <-0.6 as losses.

5. Statistical analysis

Two-sample Wilcoxon rank-sum (Mann-Whitney) test was performed to test for a difference between the average chromosome copy number/cell between the two subtypes of melanoma (SSM and NM). Fisher's exact test was used to compare the c-*myc* copy number alterations and c-*myc*/C8 copy number ratio between the number of patients with different subtypes. A *P* value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Study of c-*myc* oncogen in nodular and superficial spreading melanomas and melanoma metastases

Karyotypic abnormalities of chromosomes 1, 6, 7, 9 and 10 have been described frequently in human malignant melanoma by standard cytogenetic techniques. Recently, we found by CGH analysis that, among other chromosomal aberrations, over-representation of the long arm of chromosome 8 is more frequent in malignant melanoma than previously reported. Similar results were published by Bastian et al. reporting that 38% of primary melanomas exhibited chromosome 8q gains with the common region being the distal q arm where the c-*myc* oncogene is located. To date, only few primary melanomas were studied for c-*myc* gene copy alteration. Recently it was demonstrated by Chana et al. that the c-*myc* oncogene plays an important role in controlling melanoma growth. By using c-*myc* antisense oligonucleotides they observed the suppression of melanoma cell growth and repression of the c-myc protein expression. Their results suggest that blocking the expression of c-*myc* using an antisense approach may provide a novel gene therapy startegy for the treatment of advanced melanomas. A particular focus of the present work was to define and compare the c-*myc* gene copy number alterations in two different subtypes of cutaneous melanoma with different biological behaviour using double target FISH.

DNA probes specific for the centromeric regions of chromosomes 1, 3, 6, 7, 9 and 10 were also applied in order to characterise the ploidy level of primary and metastatic melanoma cells. In agreement with other cytogenetic and FISH studies we have found that nodular melanomas are characterised by a generally higher ploidy level compared to superficial spreading melanomas and loss of chromosome 9 is more frequent in SSMs, whereas loss of chromosome 10 is most prevalent in NMs.

Using interphase FISH, we found significant differences in the gene copy number distribution of c-*myc* between the nodular and superficial spreading subtypes of melanoma. Sixty-one percent of the NMs exhibited extra c-*myc* signals, whereas only 27% of the SSMs showed elevated number of the gene *(P* = 0.02). The difference between the c-*myc*/C8 copy number ratio, which allows for distinguishing gene amplification from polyploidy, was also significant (39% NMs and 8% SSMs, *P* = 0.01). The amplification level of the c-*myc* was generally low to moderate. We could detect relatively high level amplification (c-*myc*/C8 copy number ratio > 3) only in the nodular subtype. The elevated c-*myc* gene dosage in NMs, in association with other genetic alterations, can contribute to the invasive growth and manifest different biological behaviour.

Using FISH, we were able to compare the c-*myc* copy number changes in paired specimens (i.e. in primary tumours and their corresponding metastases). To our knowledge, no such comparison has been described before. We found that only 21% of the metastatic lesions (4 out of 14 metastases) exhibited low or moderate level of c-*myc* amplification. In 5 out of the 14 metastatic lesions studied, extra copies of the c-*myc* oncogene were present, even at higher copy number level than in their primary pairs. However, the c-*myc*/C8 ratio indicating real gene amplification was elevated only in two metastases when compared to the primary tumours obtained from the same patients. A possible explanation of this genetic divergence between the primary and metastatic tumour pairs might be the presence of heterogeneous tumour cell populations at an early stage of melanoma tumorigenesis. It is possible that, if a metastatic tumour cell escapes at a relatively early stage, cells from the metastasis and cells from the primary tumours of the same individual exhibit genetic differences. Discordance between the c-*myc* amplification patterns in primary and metastatic tumour pairs was published also for breast, lung and pancreatic cancers, describing

that, in metastatic tumours, the frequency of c-*myc* amplification is lower or absent than in the corresponding primary lesions, indicating that c-*myc* amplification is not primarily involved in the metastatic process but it may play an important role in the development of an invasive potential. Extra copies of the c-*myc* gene may result from different chromosome 8q alterations including polysomy 8, isochromosome formation and intra- or extra-chromosomal amplification of the c-*myc* locus (8q24.12-24.13). The distribution of the gene showed intratumoral heterogeneity in almost all cases, suggesting that cells in the tumour might have different growth characteristics.

The statistical analysis of our FISH data has not revealed any correlation between the patients' age and sex on comparing the incidence of c-*myc* alterations in different subtypes of primary melanoma. However, a relationship has been found between the localization of the primary tumours and c-*myc* gene copy alterations, the c-*myc*/C8 ratio being significantly higher in nodular melanomas located on the trunk or head of the patients. A weak association between c-*myc* gene copy alterations and primary tumour thickness has also been noted without any significant difference between the different thickness groups. There was a significant difference however in the proportion of patients with elevated c-*myc*/C8 ratio when the tumours were classified according to the presence or absence of ulceration in the two subtypes: the proportion of patients was significantly higher in NMs with ulcerated lesions. A relationship between an increased c-*myc*/C8 ratio and decreased disease-free interval has also been observed. Comparable data have been published by Ross et al. at protein expression level.

To date, only few, contradictory results have been published concerning the influence of c-*myc* expression on melanoma progression. Additional investigations are required to define the importance of gene amplification and increased gene and/or protein expression for the oncogenic function of the c-

myc gene in this malignancy. The critical evaluation of the c-*myc*/C8 copy number ratio, together with other markers, may allow for the identification of a subset of melanoma patients with poor prognosis. Our data indicate that the variable biological behaviour of tumour cells in superficial spreading and nodular melanomas, beside other chromosomal alterations, might be related to the different copy number patterns of the c-*myc* oncogene. Further and more detailed studies are necessary to clarify the biological significance of this oncogene as a prognostic and/or predictive marker in the management of the different subtypes of this disease.

Complex cytogenetic analysis of a novel metastatic melanoma cell line

Cell lines are useful tools in understanding the molecular genetic background of different biological behavior of malignancies. In many types of cancers, cell lines represent the most readily available models for research. By the caracterizations of our novel cell line (M35/01) established from a superficial spreading melanoma, our purpose was to describe a melanoma cell line in details using a serial of molecular cytogenetic studies.

Standard G-banding was obviously insufficient to obtain a reliable and reasonably complete pattern of genomic changes. Based on the FISH data, we suggest that the genome of M35/01 had undergone a global duplication (tetraplodization) and subsequent losses/gains. As it was shown by others, tetraploidization is an important milestone in the clonal evolution of many solid tumors.

Comparing the CGH profiles of the original tumor and the M35/01 cell line by conventional CGH, the majority of the alterations were identical in both, indicating that our cell line retained the genetic characteristics of the original tumor. Deletions of chromosome 18 and 12p12-q13.12, as well as gains on chromosome 20 could be observed only in the cell line. Losses on chromosomes 4 and 16 of the original primary melanoma included the whole

chromosomes in M35/01. There are three possible explanations for the differences observed *i.)* the cell line, similarly to the original tumor, is heterogeneous, but the ratio of cells exhibiting the observed different alterations is higher in the M35/01 cell line, *ii.)* the normal cell contamination of the primary melanoma can mask some alterations present in a small amount of cells, thus in a genetically heterogeneous tumor it may cover less dominant changes, *iii.)* it can not be excluded that alterations seen only in the cell line are the results of *in vitro* cell culturing.

Array-CGH analysis of the cell line showed similar results as got by conventional CGH analysis, representing regions that are frequently altered in melanoma, however, new alterations were also found. A relatively high amplification was observed on the 15q21-qter region. Genes on 15q15 that might be involved in melanomagenesis, include trombospondin-1 (tsp1), the product of which cooperates with the extracellular matrix and plays a role in several biological processes. In melanoma, its altered expression in the stroma is related to poor prognosis. The overrepresented region of chromosome 15 was associated with unbalanced translocations involving the short arm of chromosomes 2 and 6.

Chromosome 6 fills a special part in melanoma progression as it is supposed that both tumor suppressor genes (6q16~q23) and oncogenes may be present on this chromosome. The latter is supported by frequent amplification of chromosome 6p, although no gene related to melanoma pathogenesis could be detected so far. Amplification of the short arm of chromosome 6 and deletion of the long arm of the same chromosome has been reported both in native tissue samples and cell lines. By SKY-analysis, we observed a translocation between chromosomes 6 and 15 (der(15)t(6;15)(p21;?)). The translocated region of chromosome 6 could be identified by hybridization with band specific probes that revealed a breakpoint within 6p21. This chromosomal region harbors the gene encoding

for protein CCND3 (Ciklin-D3), the role of which in melanoma progression is poorly characterized. It was shown that, after UV irradiation, the activation of CCND3 linked CDK4 is necessary for cells to get over G2 and the initiation of mitosis and some data suggest that it could be an important prognostic indicator in superficial spreading melanoma.

Aberrations of chromosome 7 are often related to poor prognosis in melanoma, and it is supposed to be a late event. Several amplified loci could be detected by array-CGH on chromosome 7q, mainly on 7q31-q36. This region harbors *braf* oncogene the mutation of which can be found in two-third of malignant melanomas. Besides the numerical aberrations of chromosome 7, we detected a translocation between this chromosome and chromosome 17.

The most often examined and the best-known locus in melanoma progression is 9p21. Ciklin dependent kinase 2A (*p16, cdkn2, ink4, mts1*) and 2B (*ink4b, mts2, p15)* genes are located on this region. By chromosomal CGH, most part of chromosome 9 was deleted, however, by array-CGH the most affected region was 9q. The other often lost chromosome in melanoma is chromosome 10, which is similarly to chromosome 9, occur early in melanoma development. Loss of heterozygozity (LOH) studies found 10q deletion in 30-50% of primary melanomas, mainly in thinner lesions. The *pten/mmac1* gene on 10q23.3 was discovered as a tumoursuppressor gene showing mutations in high number of tumors. In more than 40% of melanoma metastases, the gene could not be detected or it was mutated. Damaged function of *pten/mmac1* was also described in gliomas, another type of cancer with neural crest origin.

Copy number changes on chromosome 16 have been reported in connection with melanoma. Herlyn et al. emphasized cadherin-expression shift during melanoma development. Expression of β_3 integrin was found to be low or absent in *in situ* melanomas and melanomas in radial growth phase, but was increased in vertical growth phase and metastatic melanomas. It was

shown that keratinocytes regulates melanocytic proliferation through direct cell-cell interaction. E-cadherin mediated adhesion seemed a critical factor between these cell types. In contrast with this observation, growth inhibition by keratinocytes did not affect melanoma cells, possibly due to the loss of contact-mediated regulation. These results support the hypothesis that escape of melanoma cells from E-cadherin-regulated control of keratinocytes might be a key event in melanoma pathogenesis.

Regarding the amplifications found in M35/01 cell line, chromosome 19 and 20 should be emphasized. Chromosome 19 together with chromosome 22 took part in chromosomal rearrangement. These chromosomes contain several known genes with possibly function in tumourigenesis, e.g. lamin-B2 (19p13), which has an essential role in the maintenance of nuclear stability and chromatin structure. Translocation of chromosome 22 to 19p13 may destroy normal gene function, thus leading to the destabilization of the nucleus and loosening chromatin structure. The alteration observed on chromosome 20 may be the result of isochromosome formation. One of the candidate genes in melanoma development on chromosome 20 is matrix metalloproteinase-9 (MMP-9, 20q11-q13). Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in metastasis formation. The enzyme encoded by this gene degrades type IV and V collagens. The MMPs' tissue inhibitor TIMP proteins (22q12) blocked MMP-9 production by melanoma cells. TIMP-1 overexpressed in B16 mouse cell line decreased tumor growth and metastasis formation.

Most of the aberrations identified in M35/01 are characteristic to superficial spreading melanomas, thus our new cell line may be a suitable tool for *in vitro* studies of this melanoma subtype.

cDNA array analyses of primary and metastatic melanomas

The advantage of microarrays containing cDNA-elements is that thousands of DNA-sequences presented as matured mRNAs can be studied in a single experiment. Although cDNA-based CGH method is not suitable to detect small intragenic mutations, they represent a new tool to eliminate the disturbing effect of normal cell contamination because even a single copy change can be detected in a sample containing as much as 60% of normal cells. By using databases containing huge amount of information, the chromosomal localizations of genes identified in these experiments can be determined easily.

By cDNA-based array-CGH, we have already analyzed 9 tumors (both primary and metastatic tumors from 4 patients and a melanoma cell line). The cDNA microarrays contained more than 12000 cDNA fragments. As for the comparison of genetic alterations between the primary and metastatic tumors it is a very efficient method to use the primary tumor as reference DNA, in case of 3 tumor pairs we used this experimental approach. According to our observations, the number of cDNA elements amplified or deleted only in the metastases, is different in the 3 analyzed cases. The less aberration was found in the tumor pair with the more advanced stage (T4bN2M0). In this case, genetic alterations had already accumulated in the primary tumor and only few new alterations were enough to the metastatic spread. When normal DNA was used as reference, in the primary tumor more than 600 cDNA elements were found to be amplified or deleted. In the metastasis from the same patient, the number of both the amplified and deleted elements was nearly doubled. These tumors were removed from a young patient, and according to the TNM classification, the primary tumor was in an advanced stage. These observations support our previous results obtained by chromosomal CGH according to which gradual accumulation of genetic aberrations is related or proportional to the aggressiveness of melanoma.

Comparing primary tumors by cDNA array-CGH, 31 know genes were found to be amplified, and 27 known genes to be deleted in all the samples. Functions of more of these genes have been already known. The most interesting is *Rho GTPase-activating protein* gene. Its decreased expression may be related to the partial or complete deletion of the gene. According to the literature, Rho proteins play an important role in the development of different cancers, and different members of the family contribute to tumor progression and metastasis formation. Analyzing the metastatic ability of melanoma cell lines, it was found that alteration of *Rho C* gene increases the motility of melanoma cells and the invasive potential of melanoma cells with minor metastatic potential. Our cDNA-based array-CGH analyses support this hypothesis.

cDNA-based array-CGH analysis of melanoma has not been published, yet, and only a BAC-based array-CGH study was reported by Harvell et al. In this investigation, the authors compared Spitz nevi and melanomas from formalin-fixed, paraffin embedded materials, and found that using array-CGH, resolution of which is better than conventional chromosomal CGH, Spitz nevi can be distinguished from malignant melanoma.

SUMMARY

1. By interphase FISH analyses we showed that primary melanomas and melanoma metastases contain heterogeneous cell populations.

2. Between nodular and superficial spreading melanomas, which have different biological behavior, we found the following chromosomal differences:
    - Except chromosome 7, in superficial spreading melanomas monosomy 7 could be observed in a significant number of tumors.
    - Polysomy of chromosomes 1, 3, 6, 7 and 8 could be observed in more nodular melanomas than in the superficial spreading subtype.
    - Polysomy 6 was found in significantly more nodular melanomas.
    - Monosomy of chromosome 9 was found more frequently in the superficial spreading subtype, while monosomy 10 in the nodular subtype.

3. We found that previously observed gain on 8q22-qter is related to the copy number increase of c-*myc* oncogene. High amplification of the c-*myc* gene could be found only in the nodular subtype.

4. The following observations were found between c-*myc* amplification and clinicopathological parameters:
    - The c-*myc*/C8 ratio was significantly higher in nodular melanomas located on the trunk or head of the patients (p=0.004).
    - There was a significant difference in the proportion of patients with elevated c-*myc*/C8 ratio when the tumors were classified according to the presence or absence of ulceration in the two subtypes: the

proportion of patients was significantly higher in nodular melanomas with ulcerated lesions (p=0.01).

- Among nodular melanomas with lymph node metastases, significantly more tumors showed elevated c-*myc* copy numbers, than among superficial melanomas (p=0.04).
- A relationship between an increased c-*myc*/C8 ratio and decreased disease-free interval was observed: c-*myc* gene amplification was more frequent among nodular melanomas of patients who died within 5 years of the surgery of their primary melanomas (p = 0.02).

5. Analyzing the new melanoma cell line established from a superficial spreading melanoma we found that:
    - Majority of the alterations of the original tumor and the cell line was the same as revealed by chromosomal CGH, indicating that the aberrations of the cell line were dominant also in the primary tumor.
    - The karyotype of M35/01 is complex, not only numerical aberrations but also several structural alterations could be detected.
    - The analysis of the cell line by centromere and gene specific probes suggests the importance of aneuploidy versus gene amplification/deletion in tumor progression.
    - The analysis of the new melanoma cell line confirms that the recognition of different genetic aberrations requires the use of more methods in parallel.

6. Amplified and deleted DNA sequences can be localized more precisely by BAC- or cDNA-based array-CGH than by chromosomal CGH.
    - By array-CGH, the highest amplifications (Log2 > 0.6) in the cell line could be detected at 7q (7q22-q31), 15q (15q21-q25), 20q (20q13), and the highest deletions (Log2 < -0.6) at 4q (4q12, 4q28-

q31, 4q32-q33), 9p (9p21, 9p23, 9p24), 9q (9q21), 10q (10q23-q25), 12q (24.3), 16q (16q13-q21, 16q21, 16q23) 18p (18p11, 18q21-23), 17p (17p12).

- Comparing primary tumors by cDNA array-CGH, 31 know genes were found to be amplified, and 27 known genes to be deleted in all the analyzed samples.

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