**DOCTOR OF PHILOSPHY (Ph.D.) THESIS WORK**

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# Evaluation of the genetic alterations associated with the progression of human malignant melanoma by *in situ* hybridization methods

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## 1. Introduction

Human malignant melanoma is the most aggressive form of skin cancer and its incidence is increasing all over the world at a rate faster than any other cancers, with the exception of lung cancer in women. The most important risk factor of melanoma is the repeated exposure to intensive UV radiation, however, other factors contribute to the risk at a lesser degree as well (e.g. type of skin, exposure to chemical carcinogens, genetic predisposition, damage to the DNA repair system, etc.). Characteristic of the natural course of melanoma is the relatively early regional and distant metastasis formation into the skin, lymph nodes and different organs involving lung, liver and bone. Patients with melanoma who develop metastases have poor prognosis, usually, they die from their disease within a few years.

It is well established that genetic alterations affecting the regulation of the cell cycle, cell growth, metabolism, apoptotic process play major role in carcinogenesis. Specific therapeutic options can be chosen on the basis of the results of molecular diagnostic tests in certain tumor types, which may be crucial for the patients. Identification of genetic alterations in malignant melanoma, therefore, is of major importance because the knowledge of the specific genetic aberrations responsible for the consequent steps of melanoma development, progression and metastasis formation may allow not only the better prediction and monitoring the disease progression but it can lead to the development of new therapeutical strategies.

In melanoma. these genetic changes are not yet well characterized, and chronology of genetic alterations has not been amenable to recent genetic analysis, so the control mechanisms of melanoma progression are still unclear. During the last decades, different approaches have been used to elucidate the genetic alterations responsible for the aggressive growth pattern and metastatic potential of this neoplasm. However, despite the substantial amount of

knowledge gathered about the genetics of melanoma from standard cytogenetic and molecular studies, the substantial genetic heterogeneity characteristic of melanoma is a major obstacle, which impedes the use of any simple genetic model to the genetics underlying the aggressive behavior of melanoma.

Classic cytogenetic analysis based on chromosome banding is often difficult, sometimes impossible and it often provides rather limited information only, which is primarily due to the technical difficulties of *in vitro* cell culture. In the last few decades molecular genetic methods not requiring *in vitro* culture of the tumor cells have become more and more important in modern cancer cytogenetics. FISH and CGH represent two powerful method of this kind. It has been shown in many cancers, CGH is one of the most useful techniques to identify chromosome regions affected by malignant transformation. It can provide fast overview of the genetic abnormalities in the scale of the whole genome and reveal chromosome regions that carry tumor specific genetic alterations. Further analysis of the suspect chromosome regions can reveal yet unknown oncogenes or tumor suppressor genes.

Although a large set of data is available for different solid tumors, only a few reports deal with the CGH analysis of human cutaneous melanomas. These studies focused only on primary tumors, and information about the genetic alterations in metastases and the potential differences is not available. Therefore, mapping the genetic alterations associated to the development and progression of malignant melanoma, and the investigation of the relationship between these genetic alterations, the aggressive metastatic behavior of the tumor and the clinico-pathologic data comprise key issue in melanoma research.

## 2. Aims

1. The aims of my work included the evaluation of the application including validation, separate and combined advantages of CGH and FISH, two modern molecular diagnostic methods in Hungary, on melanoma cell lines,

2. Analysis of genetic alterations in a rare hypodiploid primary tumor and its corresponding metastasis by FISH in order to investigate the possible genetic link as well as the genetic difference between the primary and metastatic lesions, which can lead to better understanding of genetic events of melanoma initiation and progression,

3. Analysis of genetic heterogeneity and clonal relationship in human malignant melanoma, identification and characterization of genetic alterations and chromosomal deletions-amplifications in primary tumors, melanoma metastases and in primary and metastatic lesion pairs of the same patient by CGH.

4. Malignant melanoma is prone to early metastasis and prediction of the neoplastic progression of the disease is difficult. One of the aims was to identify specific genetic alterations predisposing to or associated with the early metastatic spread of malignant melanoma, which can be used to facilitate therapeutic decision making in reasonable time.

5. Distant metastasis is responsible for the majority of melanoma death. To investigate the metastatic process in complexity, analysis of the genetic

alterations associated with distant metastasis development was performed in a melanoma liver-metastatic cell line model.

## 3. Materials and Methods

### 3.1. Melanoma tissue samples

Melanoma tumor tissues were obtained from the Department of Dermatology, University of Debrecen, Hungary. After surgical removal, the fresh tissue samples were embedded in OCT and stored at – 86 °C. The diagnosis of the tumors was carried out using hematoxylin and eosin stained tissue sections. Only specimens with a relative amount of tumor cells of at least 70% were prepared for CGH analysis. DNA was extracted according to standard protocol.

### 3.2. Cell lines

In the studies six cell lines with different metastatic capacity were used. Characterization of the cell lines was carried out by Dr. József Tímár and Dr. Andrea Ladányi (National Institute of Oncology, Budapest, Hungary).

- A2058 – Human amelanotic melanoma cell line, established from a brain metastasis developed in a 43 year-old man
- HT168 – Adaptation of A2058, the parental cell line, to in vivo growth as a xenograft resulted in the establishment of HT168 tumor and its cell line, HT168
- HT168-M1 – A highly metastatic variant selected after intrasplenic transplantation of HT168 cells into immunosuppressed mice
- WM35 – A benign, early phase (RGP) human melanoma cell line, which is not tumorigenic in nude mice
- M24 – Human melanoma cell line isolated from a lymph node metastasis which is tumorigenic in nude mice

- M24met – Aggressive cell line which was established from the metastasis of M24 cells into the lymph node of nude mice

### 3.3. Experimental melanoma liver-metastasis model

Three cell lines of the above are clonally related (A2058, HT168, HT168-M1). The HT168 cell line was derived from the parental A2058 cell line, and HT168-M1, which had aggressive metastatic potential, developed as the derivative of HT168. The liver-colonizing capacity of the HT168-M1 cells was extremely high, more than 250 liver metastases were observed after intrasplenic injection of cells immunosuppressed mice. In contrast few or no liver metastasis was observed in case of the original (A2058) and the derived HT168 tumor line under the same experimental conditions. The enhanced liver-colonizing potential of the aggressive cell line was not associated with increased in vivo or in vitro proliferative capacity.

### 3.4. Cell culture

Cells were maintained in vitro as monolayers in RPMI 1640 medium supplemented with 10% fetal calf serum at 37 °C and harvested by using 0.2% trypsin/EDTA, washed with standard PBS solution and divided into two parts to prepare genomic DNA for CGH analysis and interphase nuclei for FISH. Metaphase chromosome spreads for painting probe hybridization were obtained according to standard protocol and stored at – 20 °C.

### 3.5. Comparative genomic hybridization

#### 3.5.1. Hybridization

Hybridizations were performed according to the protocol of Kallioniemi et al. with slight modifications. Tumor and normal DNA was extracted using phenol:chloroform:isoamylalcohol after proteinase K treatment. Normal DNA was isolated from the peripheral mononuclear cells of a healthy male donor. Tumor and normal DNA was directly labeled with fluorescently labeled dUTP by nick-translation. Tumor DNA was labeled with dUTP with green fluorescence and normal DNA was labeled with dUTP with red fluorescence. Experimental conditions were adjusted to allow obtaining DNA fragments between 300-2000 bp. Nuclei were stained with DAPI dissolved in anti-fade solution. One negative control (differently labeled normal versus normal DNA) and a positive control (SpectrumGreen-dUTP labeled, cytogenetically well-characterized breast cancer cell line) were included in hybridization to validate the CGH results.

#### 3.5.2. Digital image analysis

A multicolor quantitative image processing system connected to a Zeiss fluorescence microscope was used for the acquisition of the hybridized metaphases. Six to ten images were acquired using a monochrome charge coupled device. After automatic background subtraction, chromosome segmentation and identification was performed by thresholding the DAPI image. Chromosomal alterations were evaluated on the basis of the green to red fluorescence intensity ratio profiles that were calculated for each metaphasis separately. The individual ratio profiles were combined to obtain the averaged ratio profiles. DNA gain was defined when hybridization resulted in green to red

fluorescence intensity ratio of >1.15. A loss of DNA sequences was determined when hybridization resulted in green to red ratio of <0.85. Ratio changes at the heterochromatic regions or the p-arms of acrocentric chromosomes were excluded from the analysis because these regions were blocked with Cot-1 DNA, resulting in very low signal intensities.

### 3.6. Fluorescence in situ hybridization

#### 3.6.1. DNA-probes, labeling and signal detection

In the studies we used centromere and locus specific DNA probes for chromosomes 1, 6, 7, 8, 9, 10, 11, 12, 15, 17 and X, and a painting probe was used for the entire chromosome 4. The probes were prepared by nick translation with directly or indirectly labeled nucleotides. Biotinylated and digoxigenin labeled DNA probes were visualized by Texas-Red- or FITC-avidin and FITC or TRITC labeled anti-digoxigenin monoclonal antibodies, respectively.

#### 3.6.2. Hybridization

Hybridization was performed according to standard protocol with slight modifications. Briefly, cells fixed and attached to slides were incubated in denaturing solution at 75 °C for 2-2.5 minutes, dehydrated in graded cold ethanol and air dried. Centromere specific probes and unlabeled human placental DNA were dissolved in the hybridization mixture, denatured at 73 °C for 5 minutes and applied to slides. Hybridization was performed overnight at 37 °C in a humid chamber, then the slides were washed and analyzed in the microscope.

### 3.7. Fluorescence microscopy

FISH samples were scored for the number of signals by using a Zeiss Axioplan fluorescent microscope equipped with 100 × (NA 1.3) oil-immersion objective. Fluorescent images were captured and analyzed by an image analysis system from Metasystems. Scoring was based on the method in the literature, approximately 100-200 nuclei were scored for each sample and cells were grouped by their fluorescent signal count. Each hybridization was accompanied by control hybridization by using normal lymphocytes from a healthy human donor. In case of hybridization with chromosome 4 painting probe, a total of 60 metaphases were evaluated for the presence of the fluorescent signals.

### 3.8. Measurement of DNA content by flow cytometry

Determination of the cells DNA content was performed according to a protocol in the literature. Trypsin treated cells were washed in cold PBS, centrifuged and fixed in 70% ethanol for 1 hour at 4 °C. Cells were treated with 50 μg/ml RNAse at 37 °C for 1 hour and nuclei were labeled by 10 mg/ml propidium-iodine. DNA index was determined by Betcon-Dickinson FACScan flow cytometer using CellFit software.

### 3.9. Immunohistochemistry

Trypsin treated cells were washed twice in cold PBS, resuspended in 50 μl PBS and incubated with FITC labeled monoclonal anti-EGFR antibodies for 1 hour. Cells were washed in PBS and in 1% formaldehyde/PBS solution (pH:7.00), fixed and samples were analyzed in a fluorescent microscope.

### 3.10. Statistical analysis

Contingency table analysis was used to study the relationship between chromosomal alterations and clinico-pathologic parameters (tumor size, age of patients, time elapsed till the diagnosis of metastasis, etc.) and Student t-test, ANOVA and Fisher's exact test were applied to make comparisons. Differences were defined statistically significant when p was equal to or smaller than 0.05.

## 4. Results

1. Three cell lines of melanoma origin were studied by CGH and FISH, each had different metastatic potential. By CGH, the largest number of genetic alterations was observed in the M24met cell line, which showed high metastatic potential. Its parental cell line (M24) showed less genetic alterations. Interestingly, many genetic alterations were found in the least metastatic WM35 cell line. All the cell lines were found to be aneuploid by interphase FISH using centromere specific probes. The degree of aneuploidy detected by FISH was less in the WM35 cell line than in the M24 and M24met cell lines, which correspond to the advancing stages of melanoma progression. We have to take into account the fact that CGH reveals the average changes in the DNA content of the cells, and DNA losses and gains correspond to a basal set of four or more chromosome copies on average. This can explain some of the discrepancy between the CGH and FISH results. Besides, there is a lack of information about the heterochromatic regions when CGH is used because those regions are blocked from hybridization. Based on the results of our experiments on melanoma cell lines, CGH seems to be suitable for the detection of the dominant genetic alterations in the cell populations, however, it has to be complemented by FISH to reveal certain aneuploid cell clones present in less than 50% among tumor cells. The use of centromere and locus specific FISH probes allows confirmation and characterization of genetic changes detected by CGH and provides estimate for chromosome copy numbers at a cell by cell basis.

2. In order to study the role of chromosomal deletions in the progression of malignant melanoma, we carried out a comparative analysis of copy number alterations in a close-to-diploid primary melanoma and its corresponding hypodiploid cutaneous metastasis using single and double target FISH. Our results show that loss of chromosomes 1, 9, 10, 15 and 17 were the most

frequent alterations in the primary tumor and these alterations became more expressed in the metastasis. The proportion of monosomic cell populations for chromosome 1, 9, 10, 12, 15 and 17 was twice as high in the metastatic lesion as in the primary tumor, which indicate that presence of hypodiploid cell clones in the primary tumor may be predictive of the aggressive behavior of the disease. Importantly, chromosome 9, which is suggested to play important role in both the initiation and progression of melanoma, was present in only one copy in 33% of the cells of the primary tumor already, and it was practically not detectable in the metastasis.

Generally, structural chromosomal alterations and chromosomal deletions occur at early stages of tumor development, sometimes already in the initiation phase, driving the tumor into a temporary hypodiploid state. However, there is a small number of direct evidence for that because the examination of genetic alterations occurring at initiation is rather difficult. Our interphase cytogenetic study on a primary melanoma and its metastasis provide further evidence for the importance of chromosome losses not only in the progression but in the initiation of the tumor.

3. To identify, characterize and compare the chromosomal copy number alterations present in primary and metastatic malignant melanomas and to define specific genetic abnormalities in connection with the aggressive growth pattern of this neoplasm, we performed CGH on a large set of melanoma lesions. Although, primary and metastatic lesions showed similar pattern of genetic abnormalities, alterations specific for either the primary tumors or the metastases were also found. The average number of genetic alterations per case in primary and metastatic tumors were 6.3 and 7.8, respectively. Loss of DNA sequences on chromosomes 9p and 10q was a frequent finding in both types of lesions as well as DNA gains on chromosomes 1q, 6p, 7q and 8q. The frequency of DNA gain on chromosome 7q was increased in the metastases and similar

increase was observed for the deletion of chromosome 9 at both chromosome arms. High-level amplifications were mapped to 1p12-p21 and 1p22-p31 in both the primary and metastatic lesions. Chromosome regions 4q12-q13.1, 7q21.3-qter and 8q23-qter showed high-level amplifications in the primary tumors only. High-level amplification at 20q13-qter was only found in metastatic lesions.

The CGH data unequivocally demonstrated an accumulation of genetic abnormalities during the progression of melanoma. The number of genetic alterations was significantly higher in primary tumors where patients developed metastasis within one year after the surgery of the primary lesion compared to primary tumors without metastasis during this time period. Accumulation of genetic alterations is associated with and proportional to the increase in the aggressive metastatic capacity of melanoma. Genetic alterations overrepresented in the metastatic lesions may be responsible for the selection advantage of certain cell clones among the heterogeneous cell population of the primary tumor to metastatic spread. Chromosome regions, which differ in DNA copy number in primary and metastatic lesions can harbor genes important for the metastatic progression of malignant melanoma.

4. To improve therapeutic efficiency in malignant melanoma it is very important to estimate the genetic risk of metastasis. Non-metastatic tumors (i.e. primary melanomas without metastasis at diagnosis) were compared to a set of metastatic tumors (i.e. primary tumors with metastasis at diagnosis and melanoma metastases). By the elimination of large extent of the influence of tumor heterogeneity, this classification facilitates the examination of genetic alterations underlying the metastatic phenotype of melanoma. The most frequently found alterations involving DNA gains at chromosome 6p and 8q were equally present in both the metastatic and non-metastatic lesions. Based on this observation, chromosome regions at 6p and 8q probably have an influence on the proliferative and the invasive characteristics of tumor cells as well. The

most important of the genetic differences revealed in the distinction of tumors based on their biologic behavior are the DNA gains in regions at chromosome 7p and 7q (sometimes the entire chromosome 7 was involved). This genetic alteration was more frequently observed in the metastatic lesions and the difference was statistically significant. These observations are in line with the hypothesis suggesting association between amplification of chromosome 7 and the aggressive phenotype of melanoma. Abnormalities of chromosome 7 are associated with poor prognosis in other tumor types as well, which supports the hypothesis.

The frequency of DNA losses at chromosome regions 9p and 10q differed between the tumor subgroups. These two alterations were the most frequent among deletions in the metastatic lesions. Deletion of chromosome 9 and 10 are broadly accepted as early alterations that are present from the initiation to the late progression steps. This can be explained by the genetic changes in multiple tumor suppressor genes present at these chromosomes that take place at different time points.

Based on our results, amplification on chromosome 7 and deletion at chromosome 10q, in addition to the deletion at chromosome 9p, which is thought to be a sensitive prognostic factor in melanoma, are potential genetic markers in the prognostics of melanoma. These three metastasis-associated genetic markers can improve the prediction of melanoma progression because application of multiple markers proved to be more efficient.

5. A human melanoma cell line model system was investigated to identify genetic alterations associated with liver-metastatic behavior of melanoma. Three clonally related melanoma cell lines were selected based on their distinct metastatic behavior. Adaptation of A2058, the parental cell line, to in vivo growth as a xenograft resulted in the establishment of HT168 tumor and its cell line, HT168. After intrasplenic transplantation of HT168 cells into

immunosuppressed mice, a highly metastatic variant (HT168-M1) was selected. The liver-colonizing capacity of HT168-M1 cells were extremely high.

By CGH, we could detect common DNA gains characteristic for all the three cell lines involving gains of 5p, 6p22-pter, 8q21-qter and 13q. These alterations indicate conspicuous clonal relationship which is in agreement with the clonal evolution of the cell lines from A2058 through HT168 to HT168-M1. Chromosome alterations discovered by CGH only in the highly metastatic cell line, HT168-M1 were loss of chromosomes 4, 9p21.3-pter and 10p, indicating that these alterations may be crucial during metastasis formation. Hybridization with chromosome 4 painting probe showed the deletion of one entire copy in the majority of the cells (89%), the remaining cell population had intact short arm and deleted long arm. No translocation of this chromosome was present in the highly metastatic line. The FISH chromosome painting pattern was in agreement with the CGH profile of chromosome 4 and the quantitative values represented the presence of the two cell populations in the cell line.

Chromosomal alterations found in our metastatic melanoma model by CGH and FISH are in agreement with previous cytogenetic findings but also highlight new chromosomal regions associated with enhanced metastatic capacity. The loss of chromosome 4 in the highly metastatic cell line suggests that inactivation of tumor suppressor gene(s) at this region may be as important in the aggressive behavior of human malignant melanoma as in other solid tumors.

## 5. Summary

1. Based on the results of the experiments carried out on melanoma tissue samples and melanoma cell lines, CGH and FISH are sensitive and efficient methods in the detection of chromosomal aberrations in human malignant melanoma.

2. The interphase FISH studies confirmed that chromosomal deletions are involved not only in the progression but in the initiation of melanoma as well.

3. The increasing malignancy of melanoma is associated with the accumulation of genetic alterations during tumor progression. Based on the CGH data, clonal selection in the heterogeneous cell population of the primary tumor lead to the emergence of the metastatic cell clone.

4. Increased copy number of chromosome 7 in malignant melanoma is associated with poor prognosis and aneuploidy of the chromosome or amplification of genes located on chromosome 7 are probably involved in the increased metastatic potential of melanoma.

5. Deletion of chromosome 4 in a cell line model system of liver metastasis of melanoma is an important new finding, which is associated with the extremely high liver-metastatic capacity of the cell line with the most aggressive biologic behavior. This association suggests that chromosome 4 can harbor more oncogenes and tumor suppressor genes relevant for metastasis than it was previously thought.

6. Chromosome 9 and 10 or partial sequences of these chromosomes are deleted early in the course of metastatic progression and these alterations are also important in metastasis formation. Amplification of chromosome 6p and 8q occur later and primarily are involved the tumor growth and invasion. Aberrations of chromosome 7 and deletion of chromosome 4 are associated with the appearance of metastasis and development of distant liver metastases, respectively. The alterations of the chromosomes above correspond to a hypothetical progression line.

7. Based on our own results and the data in the literature, increased copy number of chromosome 7 and deletions of chromosome 9p and 10q are potential genetic markers for metastasis development.

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