

Invited Review

Use of interphase cytogenetics in demonstrating specific chromosomal aberrations in solid tumors - new insights in the pathogenesis of malignant melanoma and head and neck squamous cell carcinoma

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Summary. The detection of structural and numerical chromosomal aberrations is an important part of the characterization of tumors and genetic diseases. The direct demonstration of DNA sequences in interphase nuclei and metaphases by fluorescence in situ hybridization (FISH) has been termed interphase cytogenetics. It has been proven as a powerful technique to detect specific aberrations in a wide variety of cell types, including paraffin-embedded tissue. Nowadays a standard method in leukemia and lymphoma, interphase cytogenetics contributes mainly to the diagnosis in these tumors and helps to classify soft tissue tumors. Therefore FISH is mandatory for the choice of therapy in these tumors. In contrast to the aforementioned, up to now, the value of FISH in solid tumors is mostly limited to pure research and contributes in this way to our understanding of tumor biology. But with the use of paraffin-embedded tissue and the first results obtained, it seems very likely that a direct correlation between histological classification and cytogenetic characteristics of solid tumors can be achieved in the near future. This information might not only provide insights into tumor biology, but could also contribute to a different tumor classification, a sort of risk estimation, where we might predict the possible biological behavior of solid tumors. This could greatly influence further therapeutic decisions thus establishing the FISH technique as an indisputable part in the diagnosis of solid tumors.

Key words: Interphase cytogenetics, Melanoma, Head and neck squamous cell carcinoma

Introduction

More than ten years ago, fluorescence in situ hybridization (FISH) was established as a tool for the detection of chromosomal aberrations (Cremer et al., 1986; Pinkel et al., 1986a,b). This direct demonstration of specific DNA sequences in interphase nuclei was also named interphase cytogenetics or molecular cytogenetics and was at first limited to cell lines and to the detection of the centromeric region of the chromosomes with DNA probes hybridizing to the repetitive sequences of the alphoid DNA family (Cremer et al., 1986). With further development of the technique, the next targets were the repeated sequences in the telomeric regions at the short and long arms of chromosomes (Buroker et al., 1987; Moyzis et al., 1988), which consist, like centromeric DNA, of almost uninterrupted repetitions of monomer sequences. These provide a large hybridization target for small probes resulting in a good fluorescence signal. Even the variation in the repeat number among individuals shown by Weier and Gray (1992) does not affect the sensitivity of FISH as demonstrated in a comprehensive clinical study (Ward et al., 1993).

One of the most important advantages of interphase cytogenetics over karyotypic analysis is the possibility to work with interphase cells; that is, with native material. Metaphases and cell cultures are not required. Therefore, a direct investigation of cellular material like blood smears or tissue sections is practicable, without the risk of cell selection through culturing. In addition, the possible analysis of sub-populations facilitates the work with genetically heterogeneous tumors. All these advantages of molecular cytogenetics lead to an intensive use of this technique in the investigation of leukemia and lymphoma shortly after the first publications. One of the first applications of interphase cytogenetics was the examination of patients with sex-mismatched bone marrow transplantation (Van Dekken

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et al., 1989). The application of FISH on tumor material followed almost immediately. The detection of trisomy 12 in chronic lymphatic leukemia (Losada et al., 1991; Anastasi et al., 1992) or trisomy 8 in myeloid leukemia (Jenkins et al., 1992) marks the beginning of the area of FISH in tumor investigation. The detection of trisomy 12 in CLL is of great importance, since it is associated with a poor prognosis (Juluisson et al., 1990). Here, FISH is much faster than chromosomal banding techniques and therefore not only helps to make a diagnosis, but is also time-saving. This is an advantage in a progressive disease.

The work with interphase cells allows an investigation of morphologically intact cells, thus making possible a combination with histological techniques. In 1993 the FICTION technique, a combination of FISH and immunophenotyping was introduced by Weber-Matthiesen et al. (1993). This technique is especially useful in the investigation of tumors which contain only a small number of aberrant cells, like Hodgkin's lymphoma (Weber-Matthiesen et al., 1995). A qualitative new step in the study of tumor samples was the introduction of chromosome libraries which "paint" a whole chromosome thus allowing the detection of translocations (Cremer et al., 1988; Lichter et al., 1988; Pinkel et al., 1988). But in addition to translocations, other types of structural aberrations like inversions and deletions play an important role in cancerogenesis. They are easily detected by FISH with locus-specific probes, such as yeast artificial chromosomes (YACs) or cosmids (Lengauer et al., 1992; Poetsch et al., 1996; Weber-Matthiesen et al., 1996).

Due to the greater difficulties in working with solid tumors than with hematological neoplasms, the application of interphase cytogenetics to these tumors started much later. In the early 1990s, the first investigation of fresh solid tumor material with FISH and centromeric DNA probes was published (van Dekken et al., 1990; Arnoldus et al., 1991). Later on, the value of FISH to demonstrate chromosomal aberrations in fixed material (paraffin-embedded sections) from a variety of solid tumors has been proven (El Naggar et al., 1994; Stock et al., 1994; Ghaleb et al., 1996; Jenderny et al., 1996), especially for small aberrations like the deletion in 1p36 (Stock et al., 1994; Jenderny et al., 1996).

Numerical aberrations in malignant melanoma

Malignant melanoma has an increasing incidence in the whole world that is faster than most other cancers (Brozena et al., 1993). In spite of a variety of cytogenetic investigations (Trent et al., 1990; Grammatico et al., 1993; Ozisik et al., 1994; Thompson et al., 1995), many molecular genetic studies, and few in situ hybridization analyses (de Wit et al., 1992; Matsuta et al., 1994a,b) little has been published concerning the prognostic value of the aberrations found. Thompson et

al. (1995) pro-posed a relevance of the loss of chromosome 10 early in tumor progression. Trent et al. (1990) reported shorter survival times of patients with structural abnormalities of chromosomes 7 and 11. Therefore, the intention of our studies was to search for a genetic characterization of tumor progression in malignant melanomas. The first step to achieve this goal was the determination of certain chromosomes which are multiplied or lost during tumor development. We decided to investigate paraffin-embedded material which is an easily reached source for a great variety of melanomas with different subtypes, Clark levels and Breslow indices. By selecting not only recently embedded tumor samples, but also up to ten-years-old material, our study included patients with a long clinical follow-up. This ensured a better assessment of the impact of aberrations on tumor development and progression.

Our intention to use paraffin-embedded material led to the choice of the fluorescence in situ hybridization techniques for our investigations, since fixed tissue could not be cultured and analyzed cytogenetically. There are two possibilities in analyzing fixed tumor samples: 1) thin tissue sections can be used; and 2) cells can be disaggregated from thick paraffin sections. Both methods have advantages and disadvantages. The analysis of thin tissue sections allows a direct correlation with morphological features, but section cutting leads to a sample containing whole and partial nuclei without the possibility to distinguish between them. Here, the estimation of the real number of chromosomes or chromosomal parts is difficult and requires a costly statistical evaluation. Especially for a deletion screening this method is not suitable. The study of isolated cells circumvents this difficulty, but due to the loss of the tissue integrity there is a great danger of analyzing cells of the surrounding normal tissue. We tried to ensure the isolation of tumor cells without adherent non-neoplastic structures by the following procedure. First hematoxylin-eosin-stained slides were carefully inspected by light microscopy to identify areas which carried a sufficient amount (at least 3mm²) of tumor tissue measured by a scaled optical adjustment. This same area was then identified on an unstained 50µm dewaxed, rehydrated and airdried tissue section which was fixed in an optical installation allowing the isolation of tumor tissue under microscopical control with a canule (used for intravenous injections).

In our studies, we investigated 36 superficial spreading melanomas, 49 nodular melanomas and 64 metastases with centromeric DNA probes for the chromosomes 1, 4, 6, 7, 9, 10, 11, 12, 15, 17, 18, X and Y (Poetsch et al., 1998a,b; unpublished data). The DNA probes for chromosome 1, 6, 7, 9, and 10 were chosen because of the possible involvement of these chromosomes in development and progression of malignant melanoma (Heim and Mitelman, 1995). All other chromosomes were used to assess the ploidy level of the melanoma cells.

The ploidy level of the cells was defined by counting the number of signals of the analyzed chromosomes. We

found greater differences in the ploidy level of the two studied subtypes of malignant melanoma. Nodular melanomas showed in general a higher ploidy level than superficial spreading melanomas in the same Clark level. In addition, the ploidy level rose from Clark level I to Clark level V regardless which subtype was looked at. The highest ploidy level was usually found in metastases. This reflects an increase of aggressive behavior of the tumor cells which is correlated to more and more errors in the replication of DNA.

Since superficial spreading melanomas and nodular melanomas display significant differences in their growth behavior, we concentrated on the comparison of these subtypes in several Clark levels. The loss of chromosomes 9 could be assigned to the less aggressive subtype of malignant melanoma - the superficial spreading melanoma. It was found especially often in Clark level I and II SSMS and less frequently in Clark level III or IV SSMS, which display by definition already a vertical growth. It seems intriguing for us to speculate that there could be a genetic difference between those SSMS which are capable of vertical growth and those which are not. On the other hand this could display a change during tumor progression. Or for those SSMS with loss of chromosomes 9 it is more "difficult" to gain the ability to grow vertically. At the moment, the most important genes on chromosomes 9 seem to be the cell cycle control genes p15/p16, whose involvement in melanoma has already been proven (Maedlandsmo et al., 1996). But a deletion of these same genes has also been found in benign nevi and normal, non-neoplastic human melanocytes (Wang and Becker, 1996), which in most concepts of melanoma pathogenesis are considered as progenitor cells without the ability to dedifferentiate.

In contrast, the loss of chromosomes 10 was associated with melanomas exhibiting vertical growth. These two different numerical aberrations and their different appearance in the two investigated subtypes of malignant melanoma are another prominent example for the possibility of the FISH analysis to correlate biological behavior with genetic aberrations. From the above-mentioned results it seems possible to us that the loss of chromosomes 10 might be a characteristic of vertical growth, a characteristic that is detectable even if the shift to vertical growth might not be identifiable by histological analysis. Here, the patient may benefit in the near future from diagnostic concepts which integrate interphase cytogenetics.

The chromosomal region 1p36 in malignant melanoma

One of the most interesting chromosomal regions in malignant melanoma (and other solid tumors) is the subtelomeric region of the short arm of chromosome 1. Many studies have been able to demonstrate loss of chromosomal material in this region in melanomas (summarized by Heim and Mitelman, 1995).

The investigation of the subtelomeric region 1p36 by

FISH is more complex than the determination of numerical aberrations, because this is not the centromeric part of a chromosome with well-defined repetitive sequences. But for the marker D1Z2 in 1p36.3 another repetitive DNA probe is available, thus facilitating the analysis of this region. Our studies of the isolated cells from paraffin-embedded material showed only small differences between the hybridization efficiency and the signal intensity of this probe compared with the centromeric probes. Therefore, we have been able to produce reliable results concerning deletion of 1p36.3 in melanomas (Fig. 1), and we have been able to demonstrate that this region is connected strongly to vertical growth (Poetsch et al., 1998b, 1999b). No deletions of the marker D1Z2 could be found in SSMS, but the incidence of this aberration rises with the Clark level in nodular melanomas, reaching a maximum of 91% in metastatic melanomas derived from NMs or even SSMS. This strongly indicates one or more tumor suppressor genes in the relevant region in which deletions lead to the transformation of tumor precursor cells like atypical melanocytes to melanocytes showing vertical growth and highly aggressive biological behavior. One of the advantages of the FISH technique is the possibility to enclose the critical region of a deletion with new panels of DNA probes. So far, we have used YAC DNA probes of the regions 1p36, 1p32, 1p31 and 1p21 in cohybridization with the centromeric DNA probe of chromosome 1 or the subtelomeric probe in D1Z2 (Poetsch et al., 1999b). These investigations have revealed that the critical region of deletion in 1p36 is

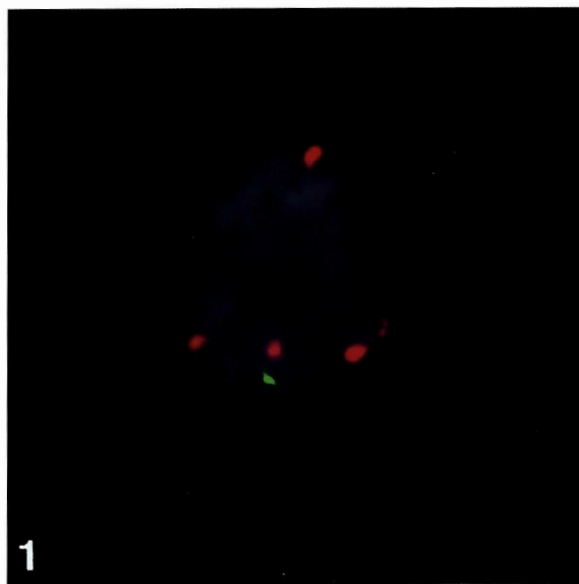


Fig. 1. Interphase cell of a nodular melanoma (Clark level IV) after hybridization with the centromeric probe for chromosome 1 (red signals) and a subtelomeric probe in 1p36 (green signal). The cell shows five signals for chromosome 1 (one subdivided into two chromatides) and only one green signal for 1p36, indicating a deletion in 1p36 in four chromosomes 1.

confined to a rather small area near the locus D1Z2. Since the newly detected tumor suppressor gene p73 in 1p36.3 (Jost et al., 1997; Kaghad et al., 1997) seems to have no part in the pathogenesis of melanoma (Kroiss et al., 1998; Schittek et al., 1999; Tsao et al., 1999), further FISH studies with YAC clones or cosmid contigs are necessary to enclose the chromosomal region for additional candidate genes with importance in this tumor.

Head and neck squamous cell carcinoma

Since we wanted to demonstrate the applicability of FISH on different solid tumors, we chose a subset of epithelial tumors as another example. Head and neck squamous cell carcinoma (HNSCC) represent a different well-known tumor besides neoplasms of the hematopoietic systems and tumors of neural crest origin like malignant melanomas.

The application of FISH on HNSCC is much more promising than the use of conventional cytogenetic analysis. This is largely due to difficulties in culturing these neoplasms, tumor heterogeneity, and often a limited number of cells analyzed (Cowan et al., 1992; Osella et al., 1992; Carey et al., 1993; Jin et al., 1993; van Dyke et al., 1994; El-Naggar et al., 1996). Therefore, already in 1993 and 1994 the first studies of FISH on head and neck cancer were published (Voravud et al., 1993; Tsuji et al., 1994). Later on, concurrent DNA flow cytometric (FCM) and FISH analyses demonstrated marked random numerical chromosomal aberration as a common feature of HNSCC,

manifestation of intertumoral and intratumoral numerical chromosomal heterogeneity in these neoplasms, significant correlation of DNA index with the net gain and loss of individual chromosomes and association of DNA ploidy status with histological differentiation and tumor stage (El-Naggar et al., 1996). Furthermore, our FISH study suggests that different tumor sites of HNSCC may also show different patterns of chromosomal changes (Poetsch et al., 1999a). In detail, the loss of chromosomes 3 and 10 may be associated with laryngeal SCC (Fig. 2), the underrepresentation of chromosomes 9 might characterize oropharyngeal tumors, and a decrease in the copy number of chromosomes 11 and 18 seems to be a feature in hypopharyngeal carcinomas. Moreover, the loss of chromosomes 18 could be connected with advanced tumor stages of all three locations (Poetsch et al., 1999a). The surgical procedure and the location seem to be important factors for the outcome in HNSCC (Grau et al., 1997; Parsons et al., 1997). Surgeons try to respect tumor-free resection margins, but, even in comparable conditions, some tumors show recurrence while others do not. Here, the investigation of the genetic background of both kinds of tumors may give further insights into the malignant capability of each tumor. In this context, it also seems of great importance to perform FISH studies on tissue slides covering the tumor and the adjacent tumor-free margins. Could it be possible that adjacent tissue, histologically determined as non-tumor epithelia, dysplastic or not, harbors genetic alterations which might be responsible for tumor recurrence? This would certainly greatly influence the surgical procedure.

In addition to the analysis of numerical chromosomal aberrations, comparative genomic hybridization (CGH) has been the method of choice for many research groups of HNSCC (Bockmuhl et al., 1996, 1998; Hermsen et al., 1997; Liehr et al., 1998), even for the differentiation between metastasizing and non-metastasizing tumors (Bockmuhl et al., 1997).

More recently, the analysis of single-copy genes by FISH has been applied on head and neck carcinomas. Lese et al. (1995) reported chromosomal localization of Int-2/Hst-1 amplification (11q13) in oral SCC detected by dual color-FISH, and proposed a key role of this event in the development and progression of these tumors. Wang et al. (1999) also investigated the chromosomal region 11q13 with an Int-2 probe. And Virgilio et al. (1996) demonstrated loss of certain exons of the FIHT gene. In our recent study, we tried to evaluate the applicability of such single-copy gene probes on isolated interphase cells of paraffin-embedded tissue (Kleist et al., 2000). Our data has given the first hints for an association between the infection with HPV viruses determined by PCR and the amplification of the Int-2 region. Besides PCR, electron microscopy, and immunohistochemistry a variety of nucleic acid hybridization techniques are available for identifying and typing of HPV infections (Roman and Fife, 1989; Syrjänen 1989, 1990). There are tests which can be

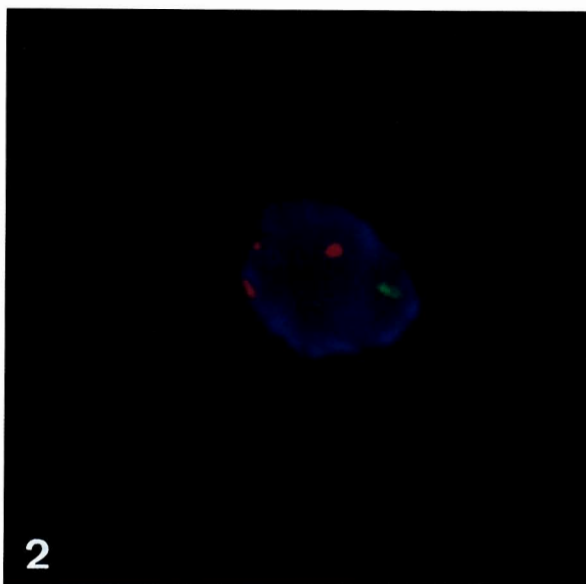


Fig. 2. Interphase cell of a moderately differentiated laryngeal carcinoma after hybridization with the centromeric probe for chromosome 1 (red signals) and the centromeric probe for chromosome 3 (green signal). The cell shows three signals for chromosome 1, but only one signal of chromosome 3, indicating a loss of chromosomes 3.

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performed directly on tissue sections, scrapes or cytological smears, e.g., ISH and FISH. They also support a role of HPV 16 in the development of a subset of oral cancers, presumably in concert with loss of function of tumor suppressor genes (Steenbergen et al. 1995).

Conclusion

The examples in this review show that FISH techniques are a useful tool for investigation of solid tumors and may provide an insight into tumor development and progression. In addition, in the near future these techniques will be an indisputable part of the diagnosis in certain solid tumors.

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