Application of specific cytologic, cytogenetic and molecular-cytogenetic techniques for the characterization of solid tumors

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Abstract

Investigations of solid tumors have shown that a very specific characterization of aberrant tissues can best be performed using a combination of cytologic, cytogenetic and molecular-cytogenetic methods. Thus, cytological analyses may serve to examine various features of tumors cultivated *in vitro*, e.g. growth peculiarities, cell morphology, specific details of cell division and mitotic rates, and anomalies of the spindle apparatus. Besides, chromosomal diagnostics characterizing non-specific aberrations focuses on the pathological karyotype and its evolution and heterogeneity, as well as on the development of secondary chromosomal aberrations. In the field of molecular-cytogenetic diagnostics we emphasize particularly the combination of metaphase and interphase analyses and the investigation of specific structural aberrations by fluorescence in situ hybridization (FISH). In contrast to the method of comparative genomic hybridization (CGH), the spectrum of applications for both methods is discussed. The findings described in this paper were obtained primarily from the analysis of 68 tumors of the urogenital tract (20 kidney tumors, 33 bladder tumors, 15 testis tumors).

Key words: tumor cytogenetics, tumor growth in vitro, polykaryotic cells, micronucleus formation, multipolar spindle apparatus, composite karyotype, meta- and interphase-FISH, CGH, cell cycle abnormalities.

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Received 22.04.2005 Accepted 26.04.2005

Introduction

Solid tumors can be analysed *in vitro* by a combination of cytologic and cytogenetic parameters. At the same time, these methods permit the differentiation between tumor cells and neighbouring normal tissue in cases where non-transformed cells may have contaminated the probe.

The object of this paper is a characterization of different cytologic and cytogenetic parameters relevant to the analysis of such tumors, in which both the potential and the limits of these methods will be discussed.

The *in vitro* solid tumor cultures described below were for the most part derived from tumors of the urogenital tract (kidney [1,2], bladder [3], and testis [4-6]). In some few cases findings obtained from head/neck tumors were included [7,8].

Cell growth and morphology

1. Cell growth

Unlimited growth is a characteristic feature of all tumor cells. Control mechanisms which, i.e. coordinate processes of proliferation in normal meristematic cells are absent.

Furthermore, a break-down of contact inhibition occurs in cell culture. Cells tend to grow in a criss-cross manner, covering each other, so that instead of a monolayer the result will be three-dimensional cell groups (*Fig. 1a-c*).

2. Cell morphology

Variable cell shape and differing nucleus-plasma ratios are a conspicuous phenomenon in tumor cells. Cytoplasmatic deposits which develop mainly around the nucleus may be attributed to a changed cell metabolism. So-called micronuclei also occur and are caused by abnormalities in the cell cycle which lead to an aberrant distribution of single chromosomes in anaphase of mitosis and the appearance of acentric fragments in the cytoplasma. These chromosomes and fragments are stimulated by the main nucleus to form an additional small nucleus.

Figure 1. **Growth abnormalities of testicular tumor cells *in vitro* (vital cell culture, phase contrast microscopy): a) criss-cross-growth, b) star-like growth, c) three-dimensional growth**

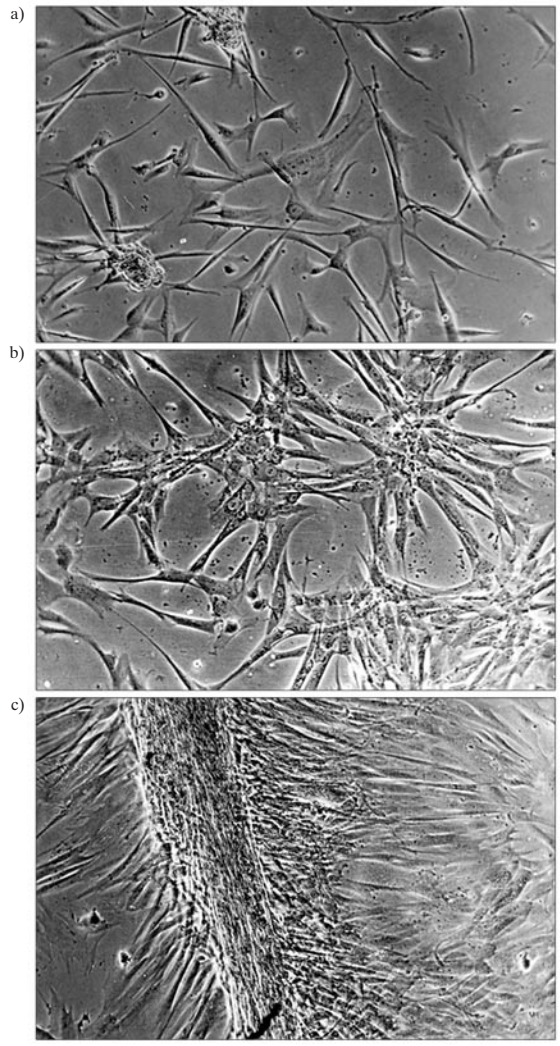
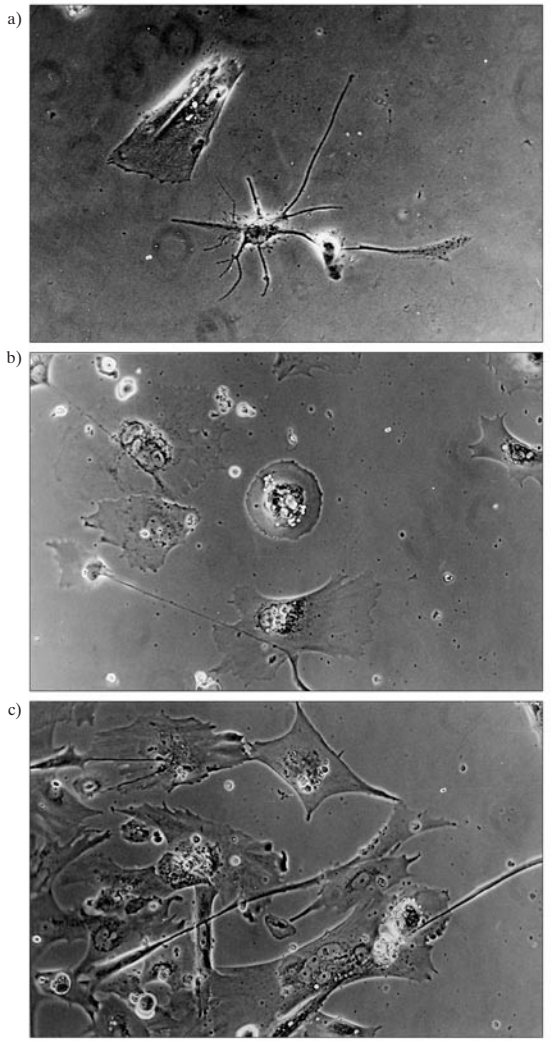


Figure 2. **Morphologic changes in testicular tumor cells *in vitro* (vital cell culture, phase contrast microscopy): a) and b) variation in cell shape, c) polykaryotic cells with perinuclear deposits**



Also multinucleated (2-8 nuclei) cells with a distinctly enlarged or diminished nucleus occur (*Fig. 2a-c*).

3. Morphology of the nucleus

Nuclei are often polymorphic, exhibiting constrictions and cytoplasmic inclusions as well as more complex structures (*Fig. 3a-c*).

4. Changes in tumor growth during prolonged or specific cultivation

Prolonged *in vitro* cultivation of tumor cells as well as the use of specific cell culture media in combination with the frequently heterogeneous nature of tumor cells may lead to the selective proliferation of particular subpopulations which may be especially well-adapted to the *in vitro* conditions, but which are not representative of the original tumor. Therefore, cultures of several different biopsy samples should be set up for each tumor culture and the results of these long-term cultures should be compared with those of a direct preparation [9,10].

5. Mitotic index and polyploidy rates

In our own studies, the mitotic index as a measure of the rate of cell division fluctuated greatly in different tumors of the same type. The mitotic index was determined two hours after addition of colcemid; the resulting arrest of cells in metaphase for an extended period of time automatically leads to an artificially increased value. Normal fibroblast cultures were used as a comparison. While controls of fibroblasts usually amount to a mitotic index of approximately 10‰, the values for tumor cells, even for the same type of tumor, varied strongly, ranging from 1-180‰. This was as well observed for the polyploidy rates, with the majority of aberrations being tetraploid mitoses. The normal reference value was 0.5-1.6%, whereas the tumor cultures showed rates of 5-10%. Isolated tumors were primarily triploid, which made their polyploidy level 6n.

6. Analysis of the spindle apparatus

The spindle apparatus may be analysed via indirect immunofluorescence, and the nucleus or rather the chromosomes are

Figure 3. **Nuclear abnormalities of testicular tumor cells *in vitro* (Acridin orange staining)**

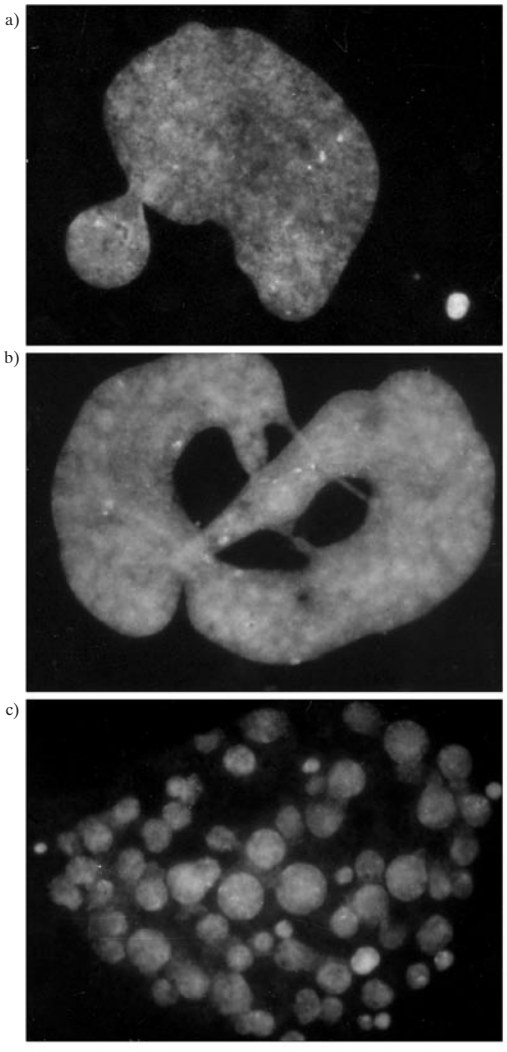


Figure 4. **Multipolar spindle apparatus (human lung carcinoma cells, cell line A549)**

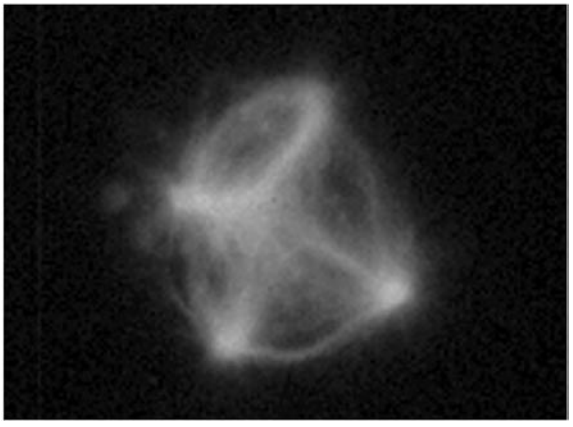
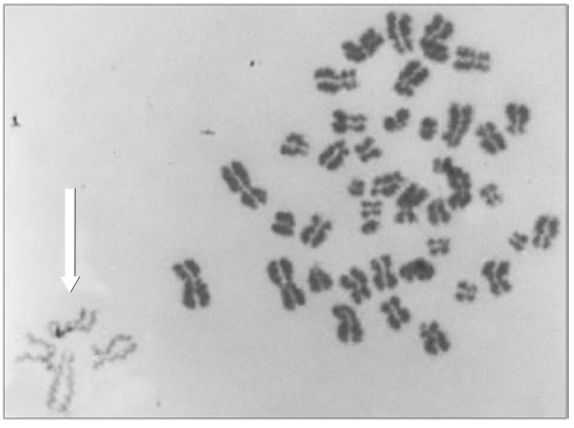


Figure 5. **Asynchronous cell cycle leading to mitosis with chromosomes in pro- and metaphase stage (Giemsa staining)**



counterstained with propidium iodid or DAPI. Typical anomalies we found in our own investigations were multipolar and degenerated spindles [11]. As before, analyses of untransformed cells served as controls. We found aberration rates of 1-2% for normal fibroblast cultures as compared to 15-40% for various tumor cultures (*Fig. 4*).

These aberrations induce a secondary increase of aneuploidy in cultured tumor cells.

Chromosome analyses

1. Non-specific chromosome aberrations

In general, chromosomes in tumor preparations exhibit a tendency to reduced condensation and aberrant spiralisation. Condensation abnormalities may be limited to isolated chromosomes. This phenomenon occurs when the chromosomes do not pass through the cell cycle in a synchronised manner. For example, individual chromosomes have not yet reached metaphase but have remained in late prophase (*Fig. 5*).

2. Karyotype analyses

In tumor cytogenetics, various banding techniques may be used, and may often be combined in order to obtain as much information as possible. This is especially necessary in cases where complex rearrangements of the chromosomes have occurred. A first analysis provides details about the modal number of chromosomes, conspicuous marker chromosomes, rearrangements and amplifications (*Fig. 6*).

3. Heterogeneity and tumor evolution

The analysis of a large number of metaphases is absolutely essential for the assessment of the heterogeneity of a cell population. Solid tumors often exhibit highly increased karyotype instability. This inherent characteristic exists *in vivo*, and persists in the *in vitro* culture as well, where new mutations can also occur. The ability of a unique karyotype to be passed on to daughter cells *in vitro* depends on the specific selection advantages and disadvantages in each case.

For tumor karyotyping it is therefore necessary to register the entirety of various characteristic aberrations and to record the tumor evolution as well.

Figure 6. **Composite karyotype of a permanent cell line (T966) of a head-neck tumor (GTG-banding)**

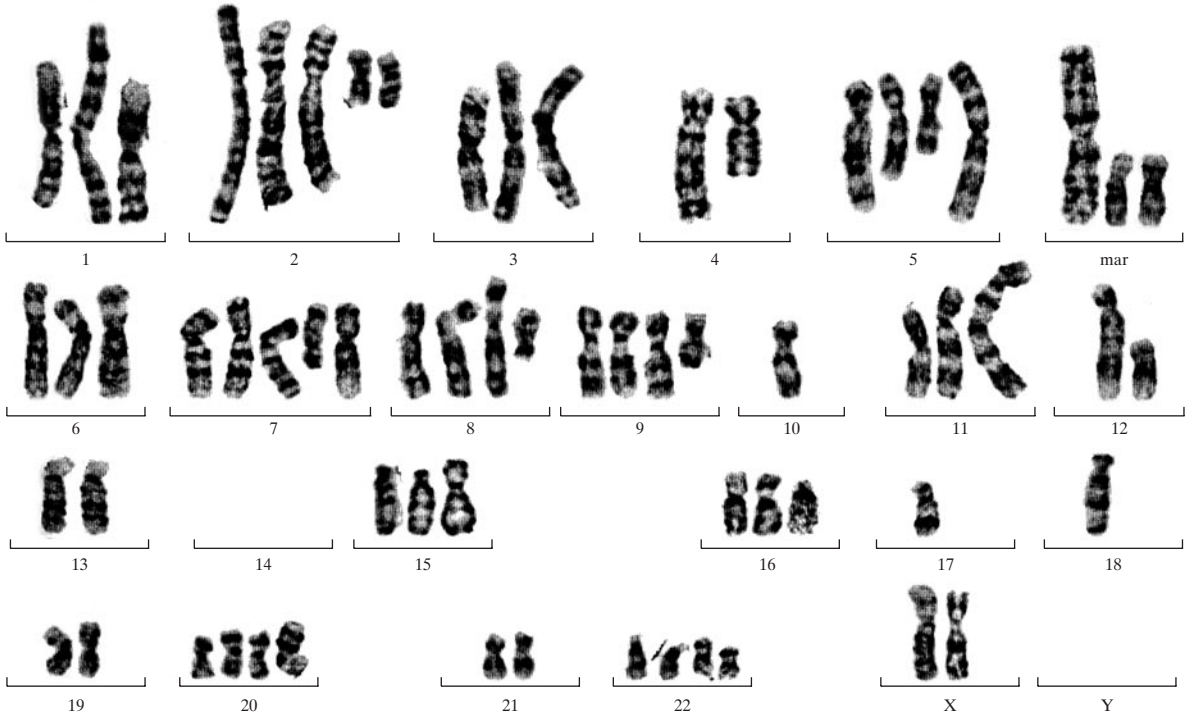


Table 1. **Results of karyotype analysis after long-term cell culture of a bladder tumor (number of analysed cells in square brackets)**

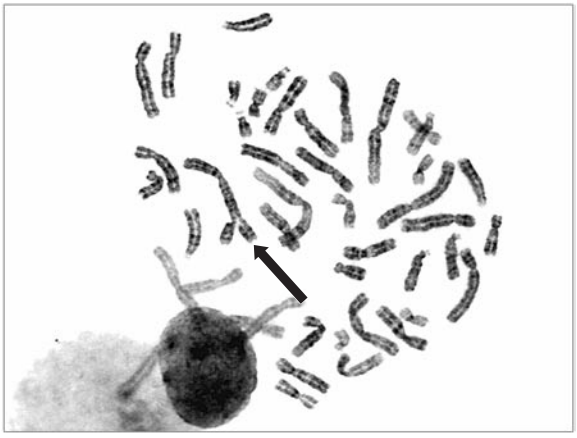
Normal karyotype:
46, XY [12]

Single karyotypes:
38, XY,-?5,-7,-11,-12,-15,-19,-19,-20
45, Y,-X
45, XY,-21 [2]
45, XY,-11
45, XY,-19
45, XY,-22
45, XY,-2,-19,+mar1
45, XY,-21,+?
46, XY,-7,+mar2
46, XY,-8,+mar3
46, XY,der(12)add(12(p13)
46, Y,-X,+7,-16,+17
85, XY,-3,-8,-14,-?15,-18
88, XXY,-6,-7,-8
89, XXYY,-?8,-13,-18
90, X,+del(4)(p?23),-5,-5,-8,+11,+11,+?14,+16,-17,t(19;?)(q?13.3;?)x2

Composite karyotypes:
Mainline: 38~90, XY,-7,-8,-19 [8]
Sideline: 45, XY,-21 [3]

A so-called "composite karyotype" contains all the clonal alterations that occur (*Tab. 1*). These can subsequently be analysed regarding their interchromosomal and intrachromosomal distribution for both numerical and structural aberrations. Furthermore, various types of aberrations (e.g. translocations, isochromosomes, deletions, duplications, monosomies, trisomies) are characteristic for distinct tumor types and for individual stages. The characterization of complex chromosomal rearrangements may require additional molecular cytogenetic analysis (*Tab. 1*).

Figure 7. **Partial endoreduplication of chromosome 2 (GTG banding)**



4. Failure of chromosomal replication and secondary aberrations

Tumor cells exhibit an increased tendency to abnormal replication of the chromosomes during S-phase of the cell cycle. This can result in complete or partial endoreduplications in the following mitosis (*Fig. 7*). Moreover, metaphases of tumor cells may show elevated rates of chromosomal breakage. In parallel, interphase cells in these cultures often display an increased rate of micronuclei (*Fig. 8*). Usually it is not possible to differentiate

Figure 8. **Interphase cell with micronucleus formation (Giemsa staining)**

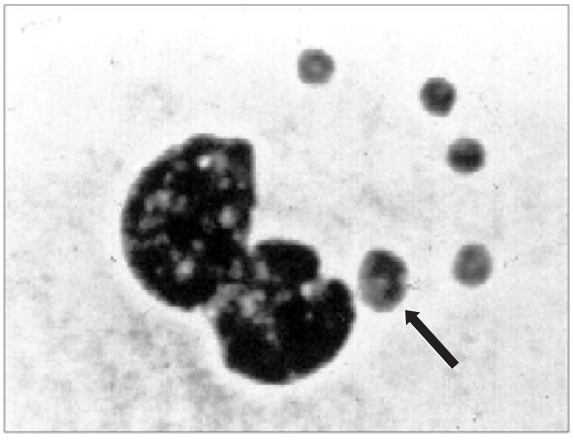
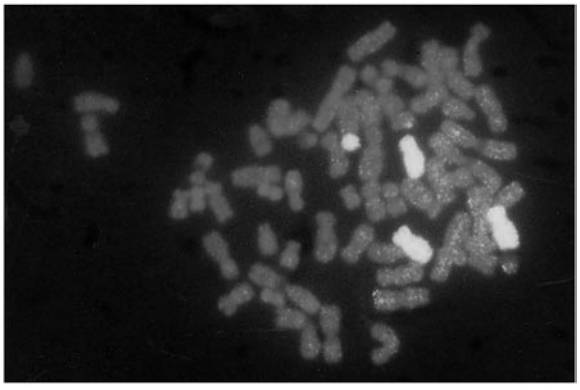


Figure 9. **Testicular tumor with an additional isochromosome of the short arms of chromosome 12 (fluorescence *in situ* hybridisation, FISH, with whole chromosome paint for chromosome 12)**



between exogenous induced breaks caused by cytostatic therapy on the one hand, and breaks caused by a higher mutation rate in the tumor on the other hand. The latter explanation appears to be more relevant, as tumor cells of patients not undergoing therapy also exhibit higher breakage rates.

Molecular cytogenetic analyses

The following is a short description of the two methods most often utilised for diagnostic purposes in tumor cytogenetics.

1. Fluorescence *in situ* hybridization (FISH)

Hybridization with defined DNA probes can be performed using metaphase or interphase spreads [12]. This technique is frequently used to characterise structurally altered chromosomes in more detail and for additional quantitative analyses (*Fig. 9a-c*). FISH is especially suited for the analysis of complex balanced rearrangements and in cases where the same chromosome occurs monosomic, trisomic or in higher amplification numbers (*Fig. 10*).

Figure 10. **Interphase analyses showing simultaneous gains and losses of chromosome X (FISH with a centromeric probe for the X chromosome)**

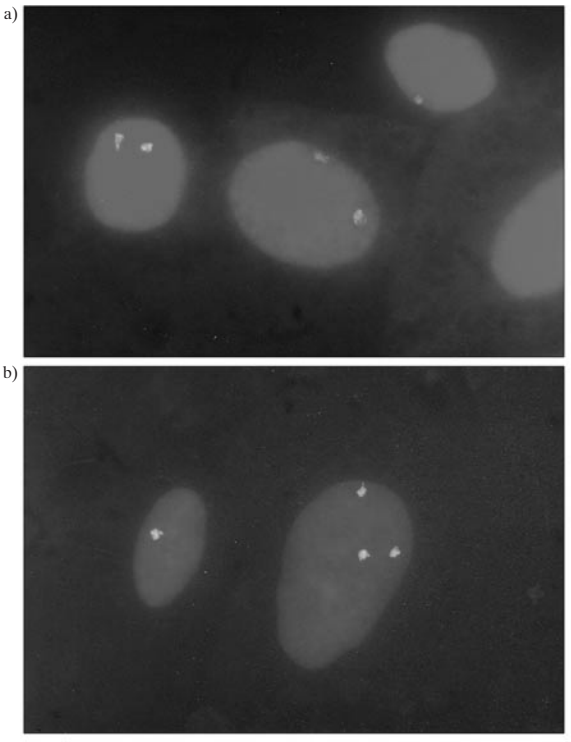
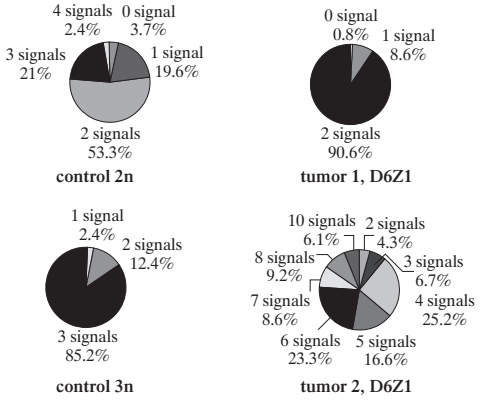


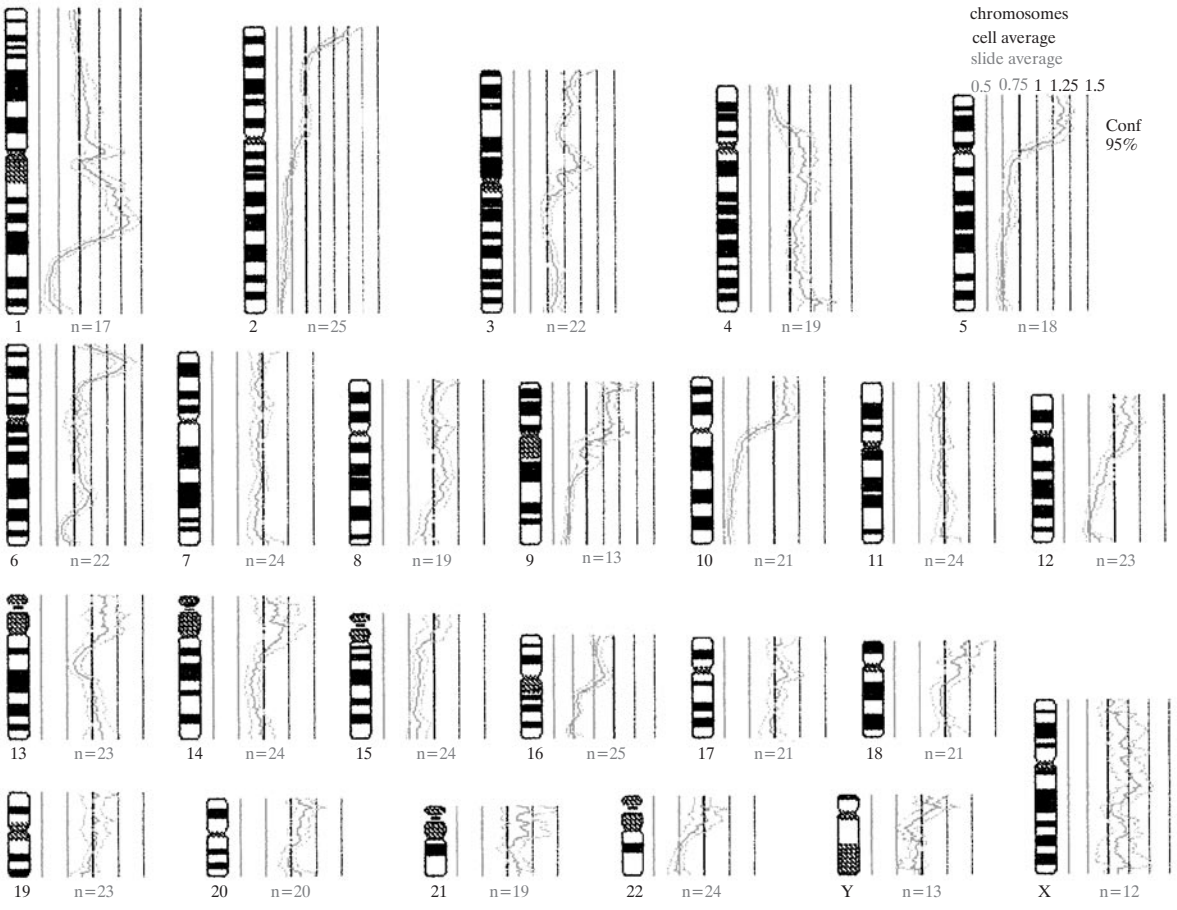
Figure 11. **Results of Interphase-FISH in head-neck tumors and control cultures demonstrating the quantitative analyses of gains and losses of chromosome 6 (FISH with a centromeric probe for chromosome 6)**



2. Comparative genomic hybridization (CGH)

This method has the great advantage to be independent from cell division stages for chromosome analysis. DNA extraction of the tumor which is to be examined can even be performed with a sample in fixative. During hybridization, chromosomal gains and losses can be determined in one single step (*Fig. 11*).

Figure 12. **CGH-profile of bladder tumor. More losses than gains of chromosomes 8, 9, 15, 17, 19, 20, 22, X and Y. More gains of chromosomes 4, 5, 11, 14, 16 and 18**



The disadvantage of this method lies in the fact that complex aberrations, the simultaneous presence of duplications and deletions and the karyotype complexity of main and side lines cannot be assessed.

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