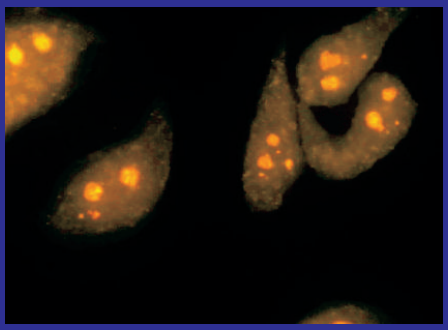
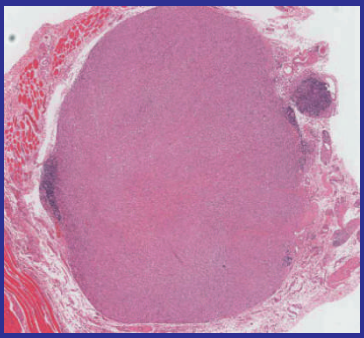
In situ hybridization of the feline major satellite DNA FA-SAT in feline fibrosarcoma cell lines and feline fibrosarcoma tissue sections





Inaugural-Dissertation
zur Erlangung des PhD-Grades
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der Justus-Liebig-Universität Giessen

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Inaugural Dissertation
Submitted to the
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in partial fulfillment of the requirements
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of the Justus Liebig University Giessen

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I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Alejandro Alfaro

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Abbreviations

AFIP	Armed Forces Institute of Pathology
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
enFeLV	endogenous feline leukemia virus
FA-SAT	feline major satellite DNA
FeSV	feline sarcoma virus
FeLV	feline leukemia virus
FeFV	feline foamy virus
FISH	fluorescence in situ hybridization
FIV	feline immunodeficiency virus
H&E	hematoxylin and eosin
ISAF	injection site-associated fibrosarcoma
IPTG	isopropyl-β-D-thiogalactopyranoside
No.	number
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
SSC	standard saline citrate
TAE	tris acetate EDTA
TBE	tris borate EDTA
WHO	World Health Organization
X-Gal	5-brom-4-chlor-3-indoxyl-β-D-galactopyranoside

1. Introduction

Fibrosarcomas are mesenchymal malignant tumors arising from fibroblasts that show no other evidence of cell differentiation (Scott et al., 2001; Ginn and Mansell, 2007).

Feline fibrosarcomas have received a great deal of attention in veterinary literature and research projects over the past 10 years (Vascellari et al., 2003). They have been considered to be the most common malignant mesenchymal neoplasm in cats, and represent about 15 % to 25 % of the cutaneous tumors in this species in many surveys (Miller, 1991; Gross and Walder, 1992). They are highly invasive, rapidly growing neoplasms, with a high rate of 60-75 % of local recurrence (Hirschberger and Kessler, 2001; Hershey et al., 2005; Eckstein et al., 2009). Several types of fibrosarcoma have been reported in the cat: the virus-induced fibrosarcoma, the intraocular post-traumatic fibrosarcoma, the fibrosarcoma without known etiology, and the injection site-associated fibrosarcoma,the later being nowadays the most frequently studied variant (Schäffer et al., 1997; Goldschmidt and Hendrick, 2002; Ginn and Mansell, 2007).

The precise mechanism of tumorigenesis of this tumor has been subject of much debate, and remains still not completely understood. Several pathogenetic and etiologic hypotheses have been proposed. Among them inappropriate inflammatory or immunologic reactions leading to uncontrolled proliferation of fibroblasts, viruses and tumor suppressor genes have been postulated most frequently (Hendrick, 1998; McEntee and Page, 2001)

In this work we studied the major satellite DNA family (FA-SAT) in feline fibrosarcomas in vitro and in biopsy samples. For this purpose, in the first part of the project we performed fluorescence in situ hybridization (FISH) for the FA-SAT on five different cell lines. Four of them were feline fibrosarcoma cell lines, and one was a skin fibroblastic cell line used as normal control. In the second part of the project, the FA-SAT was hybridized to paraffin-embedded feline fibrosarcoma and normal skin biopsies.

2. Literature review

2.1 Feline fibrosarcoma

Soft tissue sarcomas in cats represent a very important group of neoplasias. This is mainly due to the high number of fibrosarcomas in this species (Kessler, 2000).

Fibrosarcoma has been considered to be the most common feline malignant mesenchymal tumor in several surveys, and its frequency increased most likely because of its association with certain vaccinations (Miller, 1991; Gross and Walder, 1992; Goldschmidt and Hendrick, 2002; Ginn and Mansell, 2007).

The overall recurrence rate of feline fibrosarcomas is 60 % to 75 %, and repeated recurrences are common. The frequency of metastasis is 11 % to 14 %, being the lung the most common site (Miller et al., 1991; Gross and Walder, 1992; Hirschberger and Kessler, 2001).

2.2 Gross morphology

Fibrosarcomas are firm, poorly circumscribed, highly invasive and often multilobular masses that measure from less than 1 cm to more than 15 cm in diameter. Capsules are usually not seen. The cut surface is gray/white and glistening, often with an obvious interwoven fascicular pattern (Goldschmidt and Hendrick, 2002; Gross et al., 2005).

2.3 Histological classification

In the literature different sub-classifications of the feline fibrosarcoma could be found. The current international standard histological classification of mesenchymal tumors of skin and soft tissue of domestic animals is provided by the WHO International Histological Classification of Tumors of Domestic Animals of 1998 (Hendrick et al., 1998). In this international histological standard is the feline fibrosarcoma sub-classified as follows:

WHO-Histological Classification of Mesenchymal Tumors of Skin and Soft Tissue of Domestic Animals

1 Tumors of Fibrous Tissue

1.2 Malignant

1.2.1 Fibrosarcoma

1.2.1.1 Feline postvaccinal

1.2.2 Myxosarcoma

In this classification the feline fibrosarcoma was separated in two big groups, one as spontaneous tumor and the second one in which an association with an injection process was made. Other authors proposed further classifications of the tumor, taking into consideration other possible types like virus-induced, intraocular post-traumatic and spontaneous without known etiology (Kessler, 2000; Ginn and Mansell, 2007).

2.3.1 Virus-induced fibrosarcoma

The virus-induced fibrosarcoma is a rare presentation of this tumor. Feline sarcoma virus (FeSV) is the cause of multicentric fibrosarcomas in young cats, usually less than 5 years of age (Cotter, 1990; Jacobs et al., 2002; Ginn and Mansel, 2007). Overall, about 2 % of spontaneous feline fibrosarcomas are associated with FeSV (Hirschberger and Kessler, 2001).

Several strains of FeSV have been identified from naturally occurring tumors, and all were defective (Cotter, 1990). The FeSV evolves from Feline leukemia virus (FeLV) by mutation and recombination with host cellular genes. FeSV is a replication-defective retrovirus because the genes coding for proteins important for the formation of new virions are defective. In order to propagate itself, it must coexist with a replication-competent FeLV. The acquisition of a cellular oncogene yields the oncogenic potential. Through a process of genetic recombination, FeSV acquires one of several cellular oncogenes such as *fes, fms,*or *fgr*. This genetic recombination produces an acutely transforming virus that often induces multiple rapidly growing fibrosarcomas after a short incubation period. FeSV is confined to the tumor and there is no evidence of cat-to-cat transmission of these viruses (Bergman, 1998; Wise and Carte, 2005).

Fibrosarcomas caused by various strains of FeSV tend to be rapidly growing, are often multiple cutaneous or subcutaneous nodules that are locally invasive and in about one third of the cases there may occur metastases to the lung and other sites. The time for tumor development after exposure is shorter in kittens than in adults (Jacobs et al., 2002; Wise and Carte, 2005; Ginn and Mansel, 2007). All affected cats are positive for FeLV, because the FeSV needs FeLV as a helper virus for its replication (Kessler, 2000).

Solitary fibrosarcomas in old cats are not caused by FeSV, and it is not likely that the virus plays any role in injection-induced sarcomas (Wise and Carte, 2005).

2.3.2 Intraocular post-traumatic feline fibrosarcoma

Intraocular post-traumatic feline fibrosarcomas are the second most common primary intraocular tumor in cats (Schäffer et al., 1997). The pathogenesis of this tumor could be related with a trauma and its subsequent inflammatory process and/or a chronic uveitis. A lenticular capsule rupture or breakdown with leaking of lenticular substance leads to an autoimmune low-grade inflammatory disease which develops over a long period of time. In addition to that, the lenticular epithelial cells undergo fibrous metaplasia and may contribute to proliferative ocular disease in ways as yet unknown (Dubielzig et al., 1990; Hirschberger and Kessler, 2001; Zeiss et al., 2003).

History and morphological features of feline ocular sarcomas are reminiscent of feline vaccine-induced sarcomas. In comparison with other fibrosarcomas in the cat, the intraocular variant is rare (Kessler, 2000).

2.3.3 Injection site-associated feline fibrosarcoma (ISAF)

The injection site-associated feline fibrosarcoma, also known as vaccination sarcoma, has been mostly associated with killed rabies and killed feline leukemia virus (FeLV) vaccines which contain an aluminum adjuvant. Likewise, feline parvo-, herpes- and calicivirus vaccination, suture material, microchip implants, and combination of adjuvants of other types have also been associated with this reaction (Hendrick et al., 1992; Hendrick et al., 1994a; Burton and Mason, 1997; Buracco et al., 2002; Vascellari et al., 2006; De Man and Ducatelle, 2007). It is now believed that vaccines are not the only agents capable of causing sarcomas at the injection site; rather, virtually anything

that produces local granulomatous inflammation has the potential to cause the development of injection site fibrosarcoma in susceptible cats (Séguin, 2002). In fact, the inflammatory response is usually one of the distinctive features of this type of tumor (Doddy et al., 1996).

Tumor types other than fibrosarcoma have also been reported at the sites of injection. The reported tumors are osteosarcoma, malignant fibrous histiocytoma, rhabdomyosarcoma, undifferentiated sarcoma, liposarcoma, and chondrosarcoma, but these are seen at decreasing frequency (Dubielzig, et al., 1993; Hendrick et al., 1994b).

The true frequency of sarcoma development after injection is unknown. Some estimates based on retrospective epidemiologic studies and surveys of biopsy specimens submitted to US diagnostic laboratories and, in conjunction with estimates of the US cat population, report a frequency estimated to be ranging from 1 case/1000 cats to 1 case/10000 cats (McEntee and Page, 2001; Morrison and Starr, 2001). It seems that the reaction to vaccines is additive and that the likelihood of sarcoma development increases with the number of vaccines given at the same vaccination site. The risk of tumor development for cats given 3 or 4 vaccines at the cervical-interscapular region could be 175 % higher than the risk for a cat not receiving vaccines at that site (Kass et al., 1993). The time for tumor development in cats after vaccination has been reported to be as short as 2 months, and as long as 3 years or even longer (McEntee and Page, 2001).

The injection site-associated fibrosarcoma has been reported to be a locally invasive and aggressive tumor with a high rate of local recurrence and only rarely developing metastases to draining lymph nodes, mediastinum and lungs (Rudmann et al., 1996; Briscoe et al., 1998; Hershey et al., 2005). It has been shown that feline injection site-associated fibrosarcomas exhibit histopathological features consistent with a more aggressive biological behavior than fibrosarcomas at sites not used for injection (Doddy et al., 1996). Furthermore, feline fibrosarcomas found in injection sites are histologically identical to those observed in previously traumatized areas (Smith, 1995).

The mean age of affected cats is slightly lower than that of cats with fibrosarcomas that are not injection-associated. The tumor has been reported in cats as young as 3 years of age, nevertheless the mean age is 8.6 years, which is still lower than the 10.2 years reported in not injection-associated fibrosarcomas (Hendrick et al., 1994b; Doddy et al., 1996). There is no sex predilection for the development of the tumor (Goldschmidt and Hendrick, 2002).

Many theories have been proposed for the pathogenesis of feline injection site-associated fibrosarcoma. The precise mechanisms of tumor development are unknown. The broadly accepted theory is that the tumor arises from an inappropriate inflammatory reaction associated with persistent injection-site induced inflammation. This reaction leads to uncontrolled proliferation of fibroblasts that undergo malignant transformation in a subset of genetically predisposed cats (Hendrick et al., 1992; Hendrick and Brooks, 1994; Madewell et al., 2001). The component in the vaccine most commonly thought to be associated with local post-vaccination inflammation is the aluminum adjuvant in the form of aluminum hydroxide or aluminum phosphate (Hendrick et al., 1992; Madewell et al., 2001; Vascellari et al., 2003). Indeed, the importance of inflammation in tumor development and progression is well established for fibrosarcoma and other malignacies (O' Byrne and Dalgleish, 2001; Banerji et al., 2007).

Furthermore, alterations in growth factor expression and oncogenes may be involved in the pathogenesis. Injection site sarcomas have been found to be immunohistologically positive for platelet-derived growth factor (PDGF) and its receptor, epidermal growth factor (EGF) and its receptor, and transforming growth factor-β, whereas non injection site fibrosarcomas are negative or only weakly positive (Hendrick, 1998; McEntee and Page, 2001; Ginn and Mansell, 2007; Kidney, 2008). McEntee and Page (2001) proposed that lymphocytes in injection site-associated fibrosarcomas may secrete PDGF to recruit macrophages, and lead to fibroblast proliferation which in turn may lead to overexpression of c-jun, a protooncogene that is associated with cellular proliferation and oncogenesis in vitro (McEntee and Page, 2001). Likewise, when growth factors are added to cultures of fibroblasts, the physical attributes of the cells change to those of neoplastic cells, suggesting an autocrine stimulatory effect. This autocrine effect has been verified in many tumors in human beings (Hendrick, 1998).

Many oncogenes cause neoplasias by coding for and causing overexpression of growth factors or their receptors. The expression of the proto-oncogen c-jun and the presence of mutations in tumor suppressor gene p53 have also been postulated as possible cofactors in tumor development (Mayr et al., 1998; McEntee and Page, 2001; Hershey et al., 2005).

In several studies, the presence of antigen or genome of polyomavirus, feline immunodeficiency virus (FIV), endogenous feline leukemia virus (enFeLV), papillomavirus, or feline foamy virus (FeFV) in vaccine site-associated sarcomas tissue was investigated. These studies suggested that none of these viruses has any direct

involvement in the pathogenesis of injection site-associated feline fibrosarcomas (Ellis et al., 1996; Kidney et al., 2000; Kidney et al., 2001a; Kidney et al., 2001b; Kidney et al., 2001c; Kidney et al., 2002).

2.3.4 Feline fibrosarcoma with unknown etiology

All those fibrosarcomas that are not related with an injection process, trauma or viral etiology are called fibrosarcomas without known etiology. This fibrosarcoma variant is much more common than the virus-induced multicentric tumors (Hendrick et al., 1994a Ginn and Mansell, 2007).

In a retrospective study performed by Hendrick et al. (1994a) it was reported that the fibrosarcomas without relation with an injection process tend to develop in older animals in comparison with the ISAF. Moreover, it has not been associated any breed or sex predisposition for this tumor variant (Kessler, 2000).

The prognosis is as for the other tumor variants guarded, with a high rate of local recurrence of 60 to 75 %. The recurrence of the tumor can occur few weeks or months after the surgical excision (Hirschberger and Kessler, 2001).

2.4 Major feline satellite DNA family (FA-SAT)

Satellite DNA consists of highly repetitive DNA sequences in mammalian genomes, and represents a high proportion of total nuclear DNA (Heartlein et al., 1988). Tandem arrays are known as satellite DNA, which is both scattered and clustered on numerous chromosomes. These areas include the centromeres and telomeres, where most genomic satellite DNA is concentrated. Many satellite DNAs are evidently the product of duplication-amplification events (Fanning, 1987).

Fanning (1987) reported the origin and evolution of a major feline satellite DNA. It is a monomer of 483bp without extensive internal homologues. Figure1 shows the FA-SAT DNA sequence reported by Fanning (1987).

```
        10         20         30         40         50         60         70         80
TCCTGACCGGCACCGTCCCTTGTGCCCTCAACTCAGCCCTTCGGGGAGACCACTGCCCTGCTGTGGGTTCGGCGCCTGGA

        90        100        110        120        130        140        150        160
ACCTCCTGGGACCTAACCCTAACCCTAACCCTACCCCGAACCGGGACCCTCCCCCGAAACCTAAGCCTGCCTCGATCCCT

       170        180        190        200        210        220        230        240
AATCCTGCATTTGAGCCGCCTTCTGGCATGGGCCCTCACTTCAGCCCTCGAGGGAGATCACGAGACCGGAGTCGCTTCGG

       250        260        270        280        290        300        310        320
CCCTGGCGACGTCCCTGGCCCGAACCCTAACCCTAGCTAAGGCTCTCCCCTCATGGGGACCCAATTCCAACGTCTGACAG

       330        340        350        360        370        380        390        400
CATCACGCGCCTGGGGTCCAGAAGCGGTGCAGGCTGACTCGGCAAGGCACCTGGAAGCTCACCATGTCGTTTCCCAGGCC

       410        420        430        440        450        460        470        480
CGGCTGCAGCTCCCGAGGAGGATTGCGGAGGCCTCCTCGACCGCGGGAGGAACAACATGTGTGGCCGTGGCAGCCAAAGAG

GGA
```

Figure 1: Major feline satellite DNA sequence reported by Fannig (1987)

It appears to comprise about 1 to 2 % of the cat genome. The satellite is composed of two portions; 30 % is a single sequence (TAACCC and its variations) that is found in many mammals, whilst the remaining 70 % consist of a unique sequence that possesses little if any internal homology (Fanning 1987).

Santos and collaborators (Santos et al., 2004) reported the chromosomal localization of the major satellite DNA family (FA-SAT) in the domestic cat. The FA-SAT is present in some telomeres and in the centromeres of 22 of the 38 chromosomes comprising the diploid feline genome.

2.5 Centromere structure and function

The mammalian centromere is a special region of the chromosome which forms the primary constriction in condensed metaphase chromosomes and is the site of sister chromatid attachment (Larin et al., 1994). This region is composed of extremely long arrays of highly repeated DNA sequences, which in human beings and primates consist primarily of tandemly arranged, multiple copies of alpha satellite DNA (Heartlein et al., 1988; Price, 1992; Shaw, 1994).

The centromere region has a multifunctional role in cell division. It is involved in the sister chromatid pairing process, the mitotic checkpoint control, and it ensures stable inheritance of the chromosome during mitosis and meiosis. Additionally, it is the site where kinetochore formation takes place which itself is a checkpoint control of

chromosomal events during mitosis (Bloom and Yeh, 1989; Mitchel, 1996; Craig et al., 1999).

2.6 Role of satellite DNA sequences for centromere function

As previously mentioned, in human beings the alpha satellite sequences are found in the centromeric region of all chromosomes and are the most abundant DNA sequence composing it (Price, 1992; Tyler-Smith and Willard, 1993; Tyler-Smith et al., 1993; Vafa and Sullivan, 1997). Considerable experimental evidence supports the notion that alpha satellite DNA is an important component of human centromeres (Tyler-Smith et al., 1998). It has been demonstrated that as little as 140 kb of alpha satellite DNA may be sufficient to confer centromere function in human cells, and a partial deletion of alpha satellite DNA is associated with centromeric inactivation (Tyler-Smith et al., 1993). In addition to that, Harrington and collaborators (1997) showed in their work how satellite DNA can provide centromeric function in artificial chromosomes and thus producing mitotically stable chromosomes, an evidence that satellite DNA itself is the functional centromere (Harrington et al., 1997). These findings address that a correlation exists between the presence of alpha satellite and the formation of functional kinetochores (Gisselsson et al., 1999). Additionally, proteins that make up the kinetochore are postulated to assemble on certain blocks of satellite DNA and hence form a series of functional kinetochore units along the DNA. The association of these functional units would then result in the formation of the kinetochore complex (Price, 1992).

In the study performed by Haaf and coworkers (Haaf et al., 1992) human alpha satellite DNA, the major centromeric satellite of primate chromosomes was transfected into African Green monkey cells. The results suggested that satellite DNA provides the primary sequence information for centromere protein binding and is necessary for at least some functional aspects of the mammalian centromere, playing a role either in kinetochore formation or in sister chromatid apposition (Haaf et al., 1992). In addition to that, the authors pointed out an association between amplified human satellite DNA and chromosomal instability (Haaf et al., 1992). Similar associations between amplified satellite DNA and chromosomal instability have been shown in human mesenchymal tumors (Gisselsson et al., 1999).

2.7 Cytogenetic abnormalities in feline fibrosarcoma

Cytogenetic data about feline fibrosarcoma is rare. In all available reports, several chromosomal abnormalities, both numerical and morphological, have been reported. In most reports the chromosome number of the tumor was higher than the normal 2n = 38. Kalat et al. (1991) reported in his study a chromosomal hyperploidy in one tumor in an adult cat. In the reported tumor all cells showed hyperploidy with 40 to 46 chromosomes per cell. Mayr et al. (1994) reported a series of chromosomal abnormalities in two fibosarcomas. They found a 20 % frequency of trisomy D1 in one of the tumors and a marker chromosome F1 in 25 % of the cells in the second tumor. A marker chromosome F1 within other morphological abnormalities was reported again by Mayr et al. (1996) in two of four other feline fibrosarcomas.

Other numerical abnormalities reported in this type of tumor revealed the presence of 69.2 % of cells with a chromosome number ranging form 51 to 64 in one feline fibrosarcoma (Mayr et al., 1991).

In a doctoral thesis running in parallel at the Institut für Veterinär-Pathologie der Justus-Liebig-Universität Giessen, Löhberg-Grüne (2009) found in five feline fibrosarcoma cell lines established at the same institution multiple numerical chromosomal aberrations. In all cell lines several numerical aberrations were found, which ranged from 27 to 144 chromosomes per cell (Löhberg-Grüne, 2009). In addition to that, Wasieri (2009) found in another doctoral work with monoclonal cell populations established from those cell lines ongoing numerical aberration ranging from 19 to 155 chromosomes per cell as well as centrosome hyperamplification in association with chromosomal instability (Wasieri, 2009).

2.8 Chromosomal instability in neoplasia

Abnormalities of chromosome number are the most common genetic aberration in malignant neoplasms. Chromosomal instability has been defined as the gain and/or loss of whole chromosomes or chromosomal segments at a higher rate, compared to normal cells, in a population of cells. It is an ongoing process that reflects the characteristic hypermutability of tumor cells (Rajagopalan et al., 2004; Gagos and Irminger-Finger, 2005; Gollin 2005).

In most cases, aneuploidy is the result of numerical chromosomal alterations. Further segmental chromosomal gains or losses come from structural chromosomal alterations,

including reciprocal and nonreciprocal translocations, homogenously staining regions, amplifications, insertions, and deletions. Structural alterations may result in a further imbalance in gene expression, resulting in chromosomal instability (Gollin, 2005). The presence of multiple rearrangements of chromosome numbers and structure are common manifestations of genomic instability encountered in mammalian tumors. Nowadays, there is growing evidence that chromosomal instability is both, an epiphenomenon and a leading cause of cancer (Gagos et al., 2005).

2.9 Molecular diagnosis of neoplasms

The morphologic diagnosis of soft tissue tumors is based on the recognition of the cell line differentiation of the tumor tissue. The architecture of the neoplastic tissue represents an essential help for its classification, especially in types with a clear cell line differentiation (Katenkamp and Katenkamp, 2009).

Fibrosarcomas can often be diagnosed on their histological appearance in hematoxylin and eosin (H&E) stained sections (Goldschmidt and Hendrick, 2002), but sometimes the fibroblastic tumor cells cannot be clearly differentiated from adjacent non-neoplastic fibroblastic tissue or the tumor borders cannot be clearly defined by this standard method.

The gold standard of morphological diagnosis is still represented by evaluation of H&E stained histological sections (Katenkamp and Katenkamp, 2009). However, modern molecular biology methods like fluorescence in situ hybridization have been developed in order to improve the diagnosis and study of tumors.

2.9.1 Fluorescence in situ hybridization (FISH) studies in neoplasms

FISH is a molecular biology method commonly used for the detection and diagnosis of different types of neoplasms and for detection of residual tumor cells. It has been developed to the point where it can be considered as a significant adjunct to other more established methods like immunohistochemistry or PCR, for detection and characterization of genetic aberrations in human cancer (Gray and Pinkel, 1992; Trask, 2002).

FISH provides a direct link between microscope and DNA sequence. This technique allows the chromosomal and nuclear locations of specific DNA sequences to be seen in

the microscope (Trask, 2002). This approach allows individual interphase tumor cells to be identified so that aberrations such as translocations, deletions, and gene amplifications can be seen in the light microscope. This is accomplished using probes for repeated sequences found at the chromosome centromeres, whole chromosome probes, and/or probes for specific aberrant sequences (Gray and Pinkel, 1992).

3 Materials and Methods

3.1 Studied Material

3.1.1 Cell lines

Five characterized and permanent cell lines were studied for the detection of the major satellite DNA by fluorescence in situ hybridization. The cell lines were established and characterized in a previous doctoral work at the Institut für Veterinär-Pathologie, Giessen (Löhberg-Grüne, 2009; Wasieri, 2009). The cell cultures consisted of four neoplastic fibroblastic cell lines developed from four different feline fibrosarcomas, and one feline dermal fibroblastic cell line established from unaltered subcutis and used as control.

Case summaries of the patients from which the cell lines were developed are shown in the annex 8.1.

The FeLV negative infection status of these cell lines was confirmed by immunohistochemistry and by polymerase chain reaction as part of another doctoral work (Löhberg-Grüne, 2009).

3.1.2 Biopsy cases

Thirty biopsy cases from 28 cats were selected from the routine diagnostic material of the Institut für Veterinär-Pathologie der Justus-Liebig-Universität Giessen for the detection of the major satellite DNA by fluorescence in situ hybridization. The selected cases were from the years 2004, 2005, 2006 and 2008 (annex 8.2).

For the cases of the year 2004, 2005 and 2006, paraffin-embedded material from the diagnostic archive was included in the study. For the cases of the year 2008 formalin fixed tissue was sectioned and routinely processed. Two of these cats (2/28) had a recurrence of the tumor (biopsies No. T6035/04 and T8904/04; T6125/05 and T8535/06).

3.2 Fixation, embedding, staining

Biopsy samples were fixed in 10 % formalin, and then routinely processed and paraffin embedded. From each tissue 3 µm thick sections were made and routinely stained with hematoxylin and eosin (H&E).

3.2.1 Selection criteria

The selected cases were all from skin and fulfilled the diagnostic criteria established in the World Health Organization (WHO) International Histological Classification of Mesenchymal Tumors of the Skin and Soft Tissues of Domestic Animals (Hendrick et al., 1998). The diagnosis was established by the diagnostic service of the Institut für Veterinär-Pathologie der Justus-Liebig-Universität Giessen.

3.2.1.2 Histologic diagnosis

The histopathological analysis for the characterization of the tumor was always performed in an H&E stained slide, following the criteria established by the WHO as above mentioned (Hendrick et al., 1998).

All biopsy cases met the following histopathologic criteria:

They were composed of nodules in the subcutaneous tissue with a diameter that went from 3 cm to 8 cm. The tumors usually had an overlying skin with adnexa (hair follicles, sebaceous glands and sweat glands), which in some cases was focally ulcerated. The neoplastic tissue consisted of relatively well circumscribed (in some cases more infiltrative), not encapsulated, densely cellular nodules surrounded by well differentiated adipose tissue. The neoplastic cells were spindle-shaped with indistinct cell borders, typically arranged in streams and bundle patterns, separated by a moderate amount of collagen and fibrovascular stroma. The nuclei were oval to elongated, with finely stippled chromatin and inconspicuous nucleoli. Mitotic figures varied, ranging from 2-6 per high power dry field.

Additionally, unaffected skin of each patient was also included.

Case summaries of the patients from which the biopsies where taken are shown in the annex 8.2.

3.3 Molecular biology methods

Several molecular biology methods were used in order to construct a FA-SAT probe for its respective fluorescence in situ hybridization in cell lines and on paraffin-embedded tissue.

3.3.1 DNA purification from tissue using the Gentra Puregene® tissue kit

The Gentra Puregene® kit (Gentra-Systems, Minesota, USA) is designed for purification of high-molecular-weight genomic DNA. The principle of the method consists in cell lyses with an anionic detergent in the presence of a DNA stabilizer. The DNA stabilizer limits the activity of intracellular DNases and also DNases found elsewhere in the environment. The obtained DNA was precipitated, washed, air dried and rehydrated with a sterile solution.
The method of DNA purification was performed according to the recommendations of the kit manufacturer. Solutions were provided by the manufacturer.

1. freeze 75 mg tissue in liquid nitrogen, grind and put on ice
2. add 3 ml „cell lyses solution", and transfer to a plastic tube
3. add 15 µl „proteinase K solution", mix, seal and incubate overnight at 55 ºC.
4. add 15 µl „RNase A solution", mix, and incubate for 15-60 minutes at 37 ºC.
5. cool sample on ice for 1 to 3 minutes, add 1 ml „protein precipitation solution", vortex for 20 seconds vigorously, centrifuge 10 minutes at 2000 x g.
6. give 3 ml isopropanol into a new 15 ml centrifuge tube, add supernatant from point 5, mix, and centrifuge 10 minutes at 2000 x g. Discard supernatant.
7. add 3 ml 70 % alcohol to the pellet, invert several times to wash it, centrifuge 1 minute at 2000 x g. Discard supernatant, air dry pellet for up to 15 minutes.
8. add 150 µl „DNA hydration solution" to the pellet, vortex 5 seconds at medium speed, rehydrate overnight at room temperature.

The measurement of the extracted nucleic acid concentration was performed by photometric analysis. For a wave length of 260 nm and a 1cm vial, an absorption of 1 is equivalent to a concentration of 50 µg/ml DNA. To estimate the purity of the nucleic acid the ratio of their absorption at 260 nm and 280 nm was determined, and should amount to approximately 1.8.

The electrophoresis allowed the estimation of the nucleic acid quality regarding their size through its separation in fragments. The visualization of the nucleic acid fragments was performed with ethidium bromide under UV-light (254-320 nm).

3.3.2 Amplification of DNA

3.3.2.1 Primer selection and PCR procedure

PCR primers were designed on the base of the cat satellite DNA family FA-SAT sequence reported by Fanning (1987) with the Gene Bank accession number: X06372. The primers were commercially synthesized by Eurofins MWG GmbH. The primer sequences, orientation and sizes are given in table 1.

Table 1: Sequence and orientation of the primers for the FA-SAT DNA

Primer	Base sequence (5´-3´)	Orientation	Primer length	Concentration
AA1-f	5´-CAC CGT CCC TTG TGC CCT CA-3´	forward	20 bp	37 pmol/µl
AA2-r	5´-TTT GGC TGC CAC GGC CAC A-3´	reverse	19 bp	37 pmol/µl

For the FA-SAT DNA synthesis, the *Taq-Polymerase*, Biotherm ™ DNA-Polymerase (NatuTec, Frankfurt) was used. The PCR reaction composition is shown in table 2.

Table 2: PCR reaction composition for the Biotherm ™ *Taq-Polymerase* (NatuTec, Frankfurt)

Reagent	Amount µl	Concentration
DEPC-H_2O	18.1	
„10x Reaction Buffer"	2.5	10 times concentrated
MgCl	1	25 mM
Forward primer	0.5	10 µM
Reverse primer	0.5	10 µM
dNTPs	1	10 mM
BioTherm™ DNA-Polymease	0.4	5U/µl
DNA	1	100 ng/µl
Volume per reaction	25	

A conventional PCR was performed in a thermocycler Gene Amp® PCR System 2400 Perkin Elmer. The PCR reaction conditions are shown in table 3.

Table 3: PRC reaction conditions for the Biotherm ™ *Taq-Polymerase* (NatuTec, Frankfurt)

Reaction	Time	Temperature in °C	Cycles
Initial denaturation	2 min 30 s	94	-
Denaturation	30 s	94	
Annealing	30 s	57	35
Extension	45 s	72	
Final extension	10 min	72	-
Cooling	∞	4	-

3.3.3 Analysis of PCR products by agarose gel electrophoresis

The PCR products were analyzed by gel electrophoresis. For this purpose 2% (w/v) agarose (Biozym, Mineapolis) was dissolved in TAE. In each slot 12 µl of PCR product mixed with 3 µl loading dye (MBI Fermentas, St. Leon-Rot) were loaded. Parallel to the products, 6 µl of pUC19/MspI DNA marker (MBI Fermentas, St. Leon-Rot) were loaded and used as DNA ladder. The electrophoresis conditions in order to obtain a good separation of the DNA bands were 60 volts for 1.5 hours. The fluorescence of the probe was observed through UV light with a transilluminator (Vilber Loumat, Torcy, France) with a 254 nm wave length.

3.3.4 Isolation and purification of the DNA fragment

In order to obtain a high amount of DNA from an electrophoretic band, the volume of the PCR product was increased up to 32 µl mixed with 5 µl loading dye. The electrophoresis conditions were 60 volts for 2 hours. The isolation of the DNA fragment was performed by exposing the agarose gel through UV-light (302-366 nm), and sectioning out the desired area with a scalpel.

The DNA purification was performed using the PCR clean-up Gel extraction NucleoSpin® Extract II kit (Macherey-Nagel, Düren). The basic principle of the method consists in the DNA binding to a silica membrane in the presence of chaotropic salt. The binding mixture was loaded directly onto NucleoSpin® Extract II columns. Contaminations like salts and soluble macromolecule components were removed by a simple washing step with ethanolic buffer „NT3". Pure DNA was finally eluted under low ionic strength conditions with slightly alkaline buffer „NE" (5 mM Tris-HCl, pH 8.5). All buffers were provided by the manufacturer.

The method was performed according to the recommendations of the kit manufacturer.

1. Gel lysis: for each 100 mg agarose gel add 200 µl binding buffer „NT", and incubate it at 50 ºC, vortex briefly every 2-3 minutes until is completely dissolved (5-10 minutes).
2. DNA binding: Load sample onto a NucleoSpin® Extract II column in a 2 ml collecting tube. Centrifuge 1 minute at 11.000 x g, discard flow-through, and put the column into a new collecting tube.

3. Silica membrane washing: Add 600 µl washing buffer „NT3 with ethanol", centrifuge 1 minute at 11.000 x g. Discard flow-through, put column into a new collecting tube.
4. Silica membrane drying: Centrifuge extraction column 2 minutes at 11.000 x g, incubate 2 to 5 minutes at 70 ºC for total removal of the ethanol.
5. DNA elution: Put extraction column into a clean 1.5 ml micro centrifuge tube. Add 15 to 50 µl prewarmed elution buffer „NE" at 70 ºC, incubate at room temperature 1 minute to increase the yield of eluted DNA, centrifuge 1 minute at 11.000 x g.

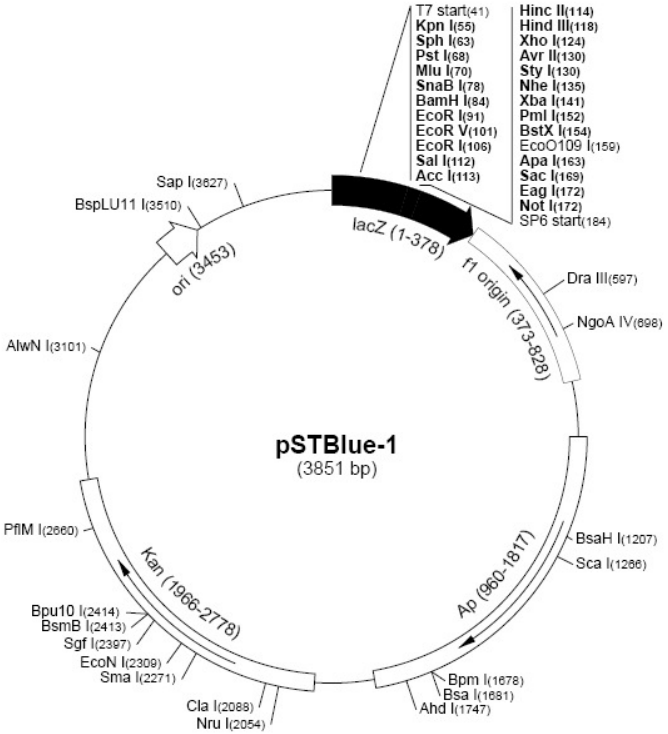
The obtained DNA was stored at -20 º C, or immediately used for following procedures.

3.3.5 Cloning of the PCR product

3.3.5.1 AccepTor™ Vector Kit

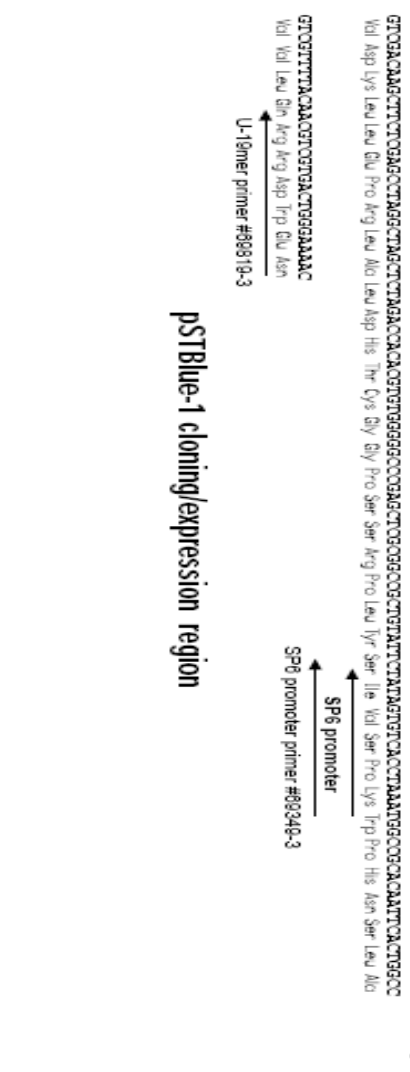
The cloning of the PCR product was performed with the help of the AccepTor™ Vector kit (Novagen, Merck, Darmstadt).

The cloning was performed using the E. coli-strain „NovaBlue GigaSingles™ competent cells" in combination with the „pST-Blue-1" vector. The pSTBlue-1 is a multi-purpose cloning vector featuring a versatile multiple cloning region, blue/white screening, dual opposed T7/SP6 promoters and dual Kanamycin/Ampicillin resistance. The linearized AccepTor™ Vector contains single 3'–dU overhangs that are compatible with direct ligation of the DNA-target insert. The restriction map, cloning site and expression region of the pSTBlue-1 vector are shown in the figure 2.



(A)

Figure 2: Restriction map (A) and cloning site and expression region (B) of the „pSTBlue-1" Vector (Novagen, Merck, Darmstadt)



(B)

Continuation of figure 2: Restriction map (A) and cloning site and expression region (B) of the „pSTBlue-1" Vector (Novagen, Merck, Darmstadt).

3.3.5.2 Ligation reaction

For a standard reaction, 1µl (50 ng; 0.02-0.022 pmol) AccepTor™ Vector was ligated with 0.15 pmol amplified product in a total volume of 10 µl. The composition of the ligation reaction mixture is shown in the table 4.

Table 4: Ligation reaction mixture

Reagent	Amount µl	Concentration
AccepTor™ Vector	1	50 ng/ µl
PCR product	0.5-4.0	0.15 pmol
Nuclease-free Water	Add up to 10	--
Clonables™ 2x ligation premix	5.0	--

Reagents of the ligation reaction mixture were gently mixed, and incubated for 2 hours at 16 ºC in order to increase the number of recombinants 2-3 times according to the recommendations of the kit manufacturer.

3.3.5.3 Transformation of the NovaBlue Singles™ competent cells

For transformation, 1 µl of the ligation reaction was added directly to the competent cells. The cells were gently mixed and incubated on ice for 5 minutes. After this incubation period, the cells were „heat shocked" by exposure to 42 ºC in a water bath for exactly 30 seconds. Then, the cells were placed on ice for 2 minutes followed by the addition of 250 µl of the provided AccepTor™ Vector kit SOC Medium (a nutrient media to enhance the transformation efficiency of the cells) at room temperature. From this solution 60 µl, 80 µl, 100 µl, twice each, and the rest of the solution were plated on agar media to ensure that one of the plates contained a sufficient number of the transformed colonies for screening. Agar plates were incubated overnight at 37 ºC.

3.3.5.4 Agar and liquid culture media

The preparation of the LB agar (annex 8.5.7) and LB liquid culture media (annex 8.5.6) was performed according to standard protocols (Sambrook et al., 1989). As the cloning vector possesses an Ampicillin resistance gene, the nutrient and the culture media on

plates were prepared with 100 µg/ml Ampicillin (Carl Roth, Karlsruhe) for selection of recombinants.

For the culture of the bacteria, the nutrient media was enriched with 40 µl IPTG-solution (23 mg IPTG in 1 ml distilled H_2O) (Carl Roth, Karlsruhe) and 40 µl X-Gal-solution (40 mg X-Gal in 1 ml DMF) (Carl Roth, Karlsruhe), and then incubated at 37 °C.

3.3.5.5 PCR of the bacterial colonies

The AccepTor™ Vectors provide a blue/white phenotype for screening of the recombinants. The plasmide encodes a functional *lacZ* α-peptide that complements the *lacZ* ω–fragment expressed by the host strain. The resulting active β-galactosidase can cleave the chromogenic substrate X-gal to yield a blue colony phenotype (colonies without insert). Inserts are cloned within the α-peptide open reading frame (ORF). Inserts disrupt this ORF, thereby preventing the production of functional α-peptide, which results in the white colony phenotype (colonies with insert) when plated on X-gal/IPTG enriched plates.

In order to verify the insert, the white colonies of the agar plates were picked with a sterile toothpick. These colonies were cultivated in 3 ml of fluid culture media and incubated overnight at 37 ºC in a shaker (Janke & Kunkel GmbH, Stauffen) at 200 rpm. Of the obtained culture 0.5 µl were transferred into a PCR-Eppendorf tube with 9.5 µl DEPC-H_2O and denatured by incubating it at 94 ºC for 10 minutes.

From the PCR master mix shown in table 5, 23 µl were added to 2 µl bacteria and a PCR was performed under the conditions shown in table 6.

Table 5: PCR master mix composition for bacterial colonies

Reagent	Amount µl	Concentration
DEPC-H_2O	288.4	
„10x Reaction Buffer"	43.6	10 times concentrated
MgCl	34.9	25 mM
Forward primer	10.9	10 µM
Reverse primer	10.9	10 µM
dNTPs	8.7	10 mM
BioTherm^{TM} DNA- Polymerase	3.5	5U/ µl
Volume per reaction	23	

Table 6: PCR reaction conditions for bacterial colonies

Reaction	Time	Temperature in °C	Cycles
Initial Denaturation	2 min 30 s	94	-
Denaturation	30 s	94	
Annealing	30 s	57	35
Extension	45 s	72	
Final extension	10 min	72	-
Cooling	∞	4	-

The results of the PCR reaction for bacterial colonies were analyzed in a 2 % agarose gel electrophoresis, as described before under point (3.3.3).

3.3.5.6 Cultivation of the appropriate bacterial colonies and isolation of the plasmid DNA

0.5 µl of the positive colonies were taken from the first culture and cultivated again in 10 ml of fluid culture medium overnight at 37 °C in a shaker (Janke & Kunkel GmbH, Stauffen) at 200 rpm.

From this culture, 4 to 6 ml were used for plasmid DNA isolation with a NucleoSpin® Plasmid Kit (Marcherey-Nagel, Düren). The basic principle of the kit consists in alkaline lysis reaction, in which the plasmid DNA is liberated from the *E. coli* host cells. The plasmid DNA binds to a silica membrane and is separated from contamination (Proteins, RNS) through several washing steps. The DNA is then eluted with the provided elution buffer.

The plasmid DNA isolation procedure was performed according to the recommendations of the kit manufacturer. All buffers were provided by the manufacturer.

1. Centrifuge 4 to 6 ml bacterial culture at 4.500 x g for 10 minutes, discard supernatant.
2. Cell lyses: Resuspend pellet in 250 µl buffer „A1" by vigorous vortexing and transfer into a 1.5 ml reaction tube. Add 250 µl lyses buffer „A2", mix carefully, and incubate at room temperature for 5 minutes. Add 300 µl neutralizing buffer „A3", mix gently, centrifuge 10 minutes at 11.000 x g at room temperature.
3. DNA binding: Load supernatant onto a silica membrane column, centrifuge 1 minute at 11.000 x g. Discard flow-through
4. Washing of the silica membrane: Add 500 µl prewarmed washing buffer „AW" at 50 ºC, centrifuge 1 minute at 11.000 x g. Discard flow-through. Second washing step, add 600 µl washing buffer „A4" with ethanol, centrifuge 1 minute at 11.000 x g. Discard flow-through.
5. Drying of the silica membrane: Centrifuge column 2 minutes at 11.000 x g. Discard flow-through.
6. Elution of DNA: Place silica membrane column in a new 1.5 ml Eppendorf tube, add 50 µl elution buffer „AE", incubate 1 minute at room temperature, centrifuge 1 minute at 11.000 x g

The obtained plasmid DNA-solution was stored at 4 °C for following experiments or at -20 °C for longer storage periods.

3.3.6 Checkup of the plasmid

The checkup of the insert in the obtained plasmid was performed by a restriction digestion reaction of the plasmid with the restriction endonuclease *Eco*RI (MBI Fermentas, St. Leon-Rot) followed by an electrophoresis analysis. The vector is designed in a way that the insert site will be flanked by the *Eco*RI recognition site, thereby – with only few additional base pairs - just the insert will be excised.
In table 7 the conditions for the digestion reaction are shown.

Table 7: Conditions for the digestion reaction of five selected bacterial colonies

Reagent	Concentration	Amount per reaction (µl) Colony number				
		1	**2**	**3**	**4**	**5**
DEPC-H_2O		14	16	16	16	16
„10 x *Eco*RI buffer"	10 x concentrated	2	2	2	2	2
*Eco*RI	10 U/l	3	1	1	1	1
Plasmid DNA	250 ng/µl	1	1	1	1	1
Total volume		20	20	20	20	20

The digestion mixture was incubated for 1 to 2 hours at 37 °C in a water-bath. After the reaction was completed, the results were analyzed in a 2 % agarose gel electrophoresis as described in 3.3.3.

3.3.7 Sequencing and analysis of the sequencing results

Sequencing of two of the obtained plasmids was performed by MWG Biotech GmbH, Ebersberg. The results were analyzed using the BLAST®-Search Program (NCBI, Bethesda, USA).

3.4 Fluorescence in situ hybridization of the FA-SAT

In situ hybridization may be defined as the direct detection of nucleic acid in cellular material in which simultaneous morphological analysis can be performed. It involves morphologically intact tissue and an hybridization process to demonstrate not only the

presence of a particular piece of genetic information but also its specific location within the tissue (Herrington and McGee, 1992; Cunningham, 1994).

3.5 Preparation of the probe

Double-stranded plasmid DNA was used as starting material for preparation of the probe. Before labeling the probe with digoxigenin, the DNA template was linearized with Eco57MI (MBI Fermentas, St. Leon-Rot), the conditions of the reaction are shown in the table 8.

Table 8: Restriction digestion with Eco57MI

Reagent	Concentration	Amount per reaction (µl)
DNA template	20 ng/µl	50
Eco57MI	2U/µl	1
10 x „Buffer B"	10 x concentrated	6
50 x Sam (S-adenosylmethionine)	1 µM	1.2
DEPC-H_2O		1.8
Total volume		60

The reaction was incubated for 1 hour at 55 ºC in a water bath. After the reaction was completed, the results were analyzed in a 2 % agarose gel electrophoresis as described in 3.3.3.

After restriction digestion, the DNA was purified with the help of a Microcon® centrifugal filter device (Millipore Corporate, Massachusetts), which allows the purification and concentration of the DNA by a centrifugation process through a filter membrane. The obtained DNA was immediately used for digoxigenin labeling.

3.6 Labeling of the FA-SAT DNA probe

The DNA labeling was performed with a DIG RNA Labeling Kit (SP6/T7) (Roche Diagnostics GmbH, Mannheim). The principle of the method consists in the production of DIG-labeled single stranded RNA probes. Either „SP6" or „T7" RNA polymerase transcribes these probes *in vitro* from the DNA template in the presence of digoxigenin-UTP producing „run off" transcripts. The DIG-UTP is incorporated into the transcript

thereby, every 20th to 25th nucleotide of the newly synthesized RNA is a DIG-UTP. The method was performed as suggested by the kit manufacturer.

1. Give 1µg purified template DNA into a sterile, RNase-free reaction vial. Adjust volume with DEPC-H_2O (annex 8.5.4) to 13 µl, and keep on ice.
2. The labeling reaction takes place by the T7 RNA polymerase transcription in the presence of digoxigenin-UTP as shown in table 9.

Table 9: Reagents for the T7 RNA polymerase transcription

Reagent	Concentration	Volume (µl)
10 x NTP Labeling mixture	10 x concentrated	2
10 x Transcription buffer	10 x concentrated	2
Protector RNase inhibitor	20 U/µl	1
RNA Polymerase T7	20 U/µl	2

Mix reagents gently, centrifuge briefly, and incubate 2 hours at 37 ºC

3. Add 2 µl of DNase I, (RNase-free) to remove template DNA, incubate 15 minutes at 37 ºC.
4. Add 2 µl 0.2 M EDTA (pH 8.0) to stop the reaction.

The labeled probe was stored at -20 ºC for following experiments.

3.7 Determination of the labeling efficiency

To determine the yield of the labeled probe, the preferred method was the direct detection via spot test. In this method, a series of dilutions of digoxigenin-labeled RNA and „labeled control RNA" (provided in the DIG RNA Labeling Kit SP6/T7) were applied to a positively charged nylon membrane. After an immunological detection and a color substrate reaction, the intensity of the control RNA used as standard and the labeled probe were compared in order to determine the labeling efficiency.

1. Apply 1 µl of five different dilutions in DEPC-H_2O (annex 8.5.4) of the digoxigenin-labeled probe and „labeled control" (pure; 1:10; 1:100; 1:1.000;

1:10.000) to a positive charged membrane Roti®-Nylon plus (Carl Roth, Karlsruhe). Dry at room temperature, fix nucleic acid to the membrane by cross-linking with UV light at 125 mjoules for 1 minute.

2. Background blocking: Incubate membrane 1 minute at room temperature in PBS (annex 8.5.11). Then, incubate 30 minutes at room temperature in „blocking solution" (1.2 ml sterile neutral sheep serum; 1.8 ml 10 % Triton-X 100; 57 ml TBS pH 7.6).
3. Immunological detection: Incubate with 3 ml 1:1.000 dilution in DEPC-H_2O (annex 8.5.4) anti-digoxigenin-AP (Fab fragments) antibody (Roche, Mannheim) 120 minutes in a dark chamber.
4. Membrane washing: 2 x 5 minutes in PBS (annex 8.5.11).
5. Color substrate reaction: Dissolve one „Fast Red tablet" in 2 ml „substrate buffer" (provided by manufacturer), apply to the membrane, incubate 15-20 minutes at room temperature.
6. Membrane washing: 2 x 5 minutes in PBS.

The color reaction produced by the „labeled control RNA" containing a concentration of 100 ng/µl was used as „standard", and was compared with the color reaction produced by the digoxigenin-labeled probe in different dilutions of both, in order to estimate its concentration.

3.8 Fluorescent in situ hybridization on cell lines

3.8.1 Preparation of chamber slides

The cell lines were cultured on a Lab-Tek® II Chamber Slide™ System (Nalge Nunc International, Naperville). Each slide is composed of four chambers, for each slide one well was always seeded with the normal skin fibroblast cell line used as a control, and the other wells contained some of the others feline fibrosarcoma cell lines.

The cells were cultured at 37 ºC in a 5 % CO_2 atmosphere until a confluent monolayer was obtained. For fixation the culture medium was removed, and the cells were washed two times in PBS (annex 8.5.11) for 5 minutes. The PBS was removed, and a freshly prepared fixation solution of 4 % formaldehyde-PBS (330 ml PBS + 40 ml 37 %

formaldehyde) was applied for 20 to 25 minutes at room temperature. The fixative was shaken off, the slides were air dried at room temperature, and stored at 5 °C until next day for FISH procedure.

3.8.2 Protease treatment of the slides

The protease treatment serves to increase target accessibility by digesting the protein that surrounds the target nucleic acid.
The protease treatment of the slides was performed as follows:

- PBS (annex 8.5.11) for 5 minutes.
- 0.2 M HCl (50 ml 2 M HCl + 450 ml bi-distilled H_2O) at room temperature for 10 minutes.
- 2 x SSC + 5 mM EDTA-Na_2 (annex 8.5.16) at 45 °C for 15 minutes twice.
- Proteinase K treatment: 2 µg/ml prewarmed proteinase K (Carl Roth GmbH, Karlsruhe) in reaction solution (annex 8.5.14), incubate 15 minutes at 37 °C.
- 0.2 % Glycine-PBS (annex 8.5.5) for 5 minutes.
- PBS for one minute twice.
- PBS + 5 mM $MgCl_2$ (annex 8.5.12) for 15 minutes.

3.8.3 Acetylation

The acetylation was performed by incubating the slides in 0.25 % acetanhydride in 0.1 M triethanolamine-DEPC-H_2O pH 7.5 (annex 8.5.4) for 10 minutes at room temperature. This step was followed by two washes of PBS for 1 minute, and 1 wash of 1 PBS for 15 minutes.

3.8.4 Pre-hybridization

A pre-hybridization incubation is often necessary to prevent background staining. The pre-hybridization mixture contains all components of a hybridization mixture except for the probe and dextran sulfate. For this purpose, the slides were incubated in prewarmed pre-hybridization solution (annex 8.5.13) at 50 °C for 1.5 to 2 hours.

3.8.5 Denaturing and hybridization

Denaturing the DNA to a single-strand state was performed by applying the probe (30 ng/slide) mixed with the „hybridization solution" (Sigma, Missouri) to the slide, and incubating it at 83 ºC for 5 minutes on a heating plate.

The hybridization depends on the ability of denatured DNA to reanneal with the complementary strand in an environment just below their melting point. After the DNA denaturing, the slides were transferred into a humid-chamber and hybridized overnight at 55 °C.

3.8.6 Post-hybridization washes

A labeled probe can hybridize nonspecifically to sequences which are partially but not entirely homologous to the probe sequence. Such hybrids are less stable than perfectly matching hybrids. They can be dissociated by performing washes of various stringencies. The stringency of the washes can be manipulated by varying formamide concentration, salt concentration, and temperature.

The posthybridization washes were performed as follows:

- 6 x SSC (standard saline citrate) with 45 % formamide (60 ml 20 x SSC + 90 ml 99.5 % Formamid + 50 ml distilled H_2O) at 42 °C for 10 minutes twice.
- 2 x SSC at room temperature for 5 minutes twice.
- 2 x SSC at 45 °C for 5 minutes.
- 0.5 x SSC at 45 °C for 15 minutes.
- 1 x SSC at room temperature for 1 minutes.

3.8.7 Blocking reaction

The blocking reaction was used prior to the immunological procedure to prevent high background by blocking non-specific antibody binding.

The reaction was performed by incubating the slides in „buffer 1" (annex 8.5.3) for 1 minute, followed by an incubation in „blocking solution" (annex 8.5.2) for 30 minutes at room temperature.

3.8.8 Immunological reaction

The immunological detection of the digoxigenin-labeled target was performed with a monoclonal antibody to digoxigenin (Anti-Digoxigenin, Roche, Mannheim). The detection of the bound antibody was performed directly with Cy3-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (Dianova GmbH, Hamburg). The immunological detection was performed as follows:

- First antibody: 1:250 dilution Anti-Digoxigenin (Roche, Mannheim), (12 µl Anti-Digoxigenin antibody + 31 µl neutral sheep serum + 94 µl 10 % Triton X 100, volume up to 3 ml with „Buffer 1" annex 11.5.3), incubate 2 hours at room temperature in the dark.
- PBS for 5 minutes at room temperature.
- Second antibody: 1:800 dilution in PBS of Cy™ 3 Goat Anti-Mouse IgG (H+L) (Dianova GmbH, Hamburg), incubated 1 hour at room temperature in the dark.
- PBS for 5 minutes twice.

3.8.9 Cell nucleus staining

In order to appreciate nucleus morphology and the localization of the FISH signals within the cell, the slides were stained with Sybr Green (Invitrogen GmbH, Karlsruhe). Sybr Green is an asymmetrical cyanine dye that binds to double stranded DNA staining it with a green color. The slides were incubated with a 1:10.000 Syber Green dilution in TBS (annex 8.5.18) for 10 to 15 minutes at room temperature. Then, the slides were washed twice in PBS for 5 minutes at room temperature. Finally the slides were covered with a cover slip using fluoromount (Serva GmbH, Heilderbeg) in order to conserve the fluorescence of the slides for longer periods.

3.9 Fluorescence in situ hybridization on paraffin-embedded tissue

FISH for the FA-SAT DNA was performed in 30 feline fibrosarcoma biopsy cases of the Institut für Veterinär-Pathologie JLU-Giessen.

3.9.1 Preparation of the tissue sections

3.9.1.1 Dewaxing of the sections

A necessary prerequisite for the analysis of archival paraffin-embedded material is the removal of paraffin wax. This was achieved by treating the slides with a series of xylene and ethanol solutions:

- Xylene 5 minutes (three times)
- Isopropanol 5 minutes
- Ethanol 96 % 5 minutes
- Ethanol 70 % 5 minutes
- DEPC-H_2O 5 minutes
- DEPC-H_2O 1 minute
- PBS 5 minutes

3.9.1.2 Protease treatment of the tissue slides

The protease treatment serves to increase target accessibility by digesting the protein that surrounds the target nucleic acid.
The protease treatment of the slides was performed as follows:

- 0.2 M HCl (50 ml 2 M HCl + 450 ml bi-distilled H_2O) at room temperature for 20 minutes.
- 2 x SSC + 5 mM EDTA-Na_2 (annex 8.5.16) at 50 °C 30 minutes twice.
- Proteinase K treatment: 20 µg/ml prewarmed proteinase K (Carl Roth GmbH, Karlsruhe) in reaction solution (annex 8.5.14), incubate 30 minutes at 37 °C.
- 0.2 % Glycine-PBS (annex 8.5.5) 5 minutes.
- PBS (annex 8.5.11) 1 minute twice.
- PBS + 5 mM $MgCl_2$ (annex 8.5.12) 15 minutes.

3.9.1.3 Acetylation

The acetylation was performed by incubating the slides in 0.25 % acetanhydride in 0.1 M triethanolamine-DEPC-H_2O pH 7.5 (annex 8.5.1) for 10 minutes at room

temperature. This step was followed by two washes with PBS for 1 minute, and 1 wash with PBS for 15 minutes.

3.9.1.4 Pre-hybridization

The tissue sections were incubated in prewarmed „pre-hybridization solution" (annex 8.5.13) at 50 ° C for 2 hours.

3.9.1.5 Denaturing and hybridization

Denaturing the DNA to a single-strand state was performed by applying the probe (30 ng/slide) mixed with the „hybridization solution" (Sigma, Missouri) to the slide, and incubating it at 83 ºC for 5 minutes on a heating plate. After the DNA denaturing, the slides were transferred into a humid-chamber and hybridized overnight at 55 °C.

3.9.1.6 Post-hybridization washes

A labeled probe can hybridize nonspecifically to sequences which are partially but not entirely homologous to the probe sequence. Such hybrids are less stable than perfectly matching hybrids. They can be dissociated by performing washes of various stringencies. The stringency of the washes can be manipulated by varying formamide concentration, salt concentration, and temperature.
The posthybridization washes were performed as follows:

- 6 x SSC (standard saline citrate) with 45 % formamide (60 ml 20 x SSC + 90 ml 99.5 % Formamid + 50 ml distilled H_2O) at 42 °C for 15 minutes twice.
- 2 x SSC 5 minutes at room temperature twice.
- 1 x SSC 15 minutes at 45 °C.
- 0.2 x SSC 15 minutes at 45 °C.
- 1 x SSC 5 minutes at room temperature.

3.9.1.7 Blocking reaction

The reaction was performed in order to prevent high background by blocking non-specific antibody binding by incubating the slides in „buffer 1" (annex 8.5.3) for 1

minute, followed by an incubation in ,,blocking solution" (annex 8.5.2) for 30 minutes at room temperature.

3.9.1.8 Immunological reaction

The immunological detection of the target was performed as described under 3.8.8

3.9.1.9 Cytoplasm and cell nucleus staining

In order to appreciate nucleus morphology, the slides were incubated with a 1:10.000 Sybr Green (Invitrogen, Karlsruhe) dilution in TBS (annex 8.5.18) for 10 to 15 minutes at room temperature. Then, the slides were washed in PBS for 5 minutes at room temperature.

For staining of the cytoplasm, the slides were incubated with an Evans Blue-PBS solution (2mg/ml Evans Blue in PBS) for 8 minutes. The slides were rinsed two times each in PBS for 5 minutes. The slides were covered with a cover slip using fluoromount (Serva GmbH, Heidelberg) for a better and longer conservation of the fluorescence.

3.10 Analysis of the FA-SAT FISH signals

Interpretation of our FISH signals was very much dependent on statistical analysis and had inherent technical challenges. Therefore, the accuracy of FISH signal analysis was dependent upon standardizing the scoring criteria accordingly (Bayani and Squire, 2004).

3.10.1 Microscopy and photography of the FISH slides

The FISH slides were analyzed using an Universal microscope Axioplan 2 (Carl Zeiss Jena GmbH, Jena) in epifluorescence. The documentation of pictures taken until the indicated number of cells was analyzed was performed using a compact digital camera (Canon Powershot A640, Canon inc.) and computer software (Axio Vision Release 4.6, Carl Zeiss MicroImaging GmbH, Göttingen). The pictures were stored on a hard drive and analyzed with Abobe Photoshop CS2 and UTHSCSA ImageTool 3.0 (University of Texas Health Science Center, San Antonio, Texas) in order to score the FISH signals.

3.11 Microscope technical specifications

The fluorescence filters used in the Universal microscope Axioplan 2 (Carl Zeiss Jena GmbH, Jena) were:

- For Cy3™ Goat Anti-Mouse IgG (H+L) (Dianova, Hambuerg): Zeiss filter 43, with excitation BP 545/25, beam splitter FT 570, emission BP 605/70.
- For Sybr Green (Invitrogen GmbH, Karlsuhe): Zeiss filter 09, excitation BP 450-490, beam splitter FT 510, emission LP 520.

3.12 Statistical analysis

In order to analyze the FISH signal pattern in cell lines and tissue sections different statistical methods were employed. A multiple comparison procedure to find whether cell lines were significantly different from one another in their total number of signals was performed with a Post-hoc-test (Nemenyi). A non-parametric test (Wilcoxon-Mann-Whitney-Test) for assessing whether the tumor tissue and control tissue were significantly different in their total number of signals per cell was performed also. For those fibrosarcomas which were recurrent, a two factorial analysis of variance of the total number of signals per cell was performed for the analysis of the first tumor and its recurrence, as well a for the first and second control skin biopsy.

The analysis of the estimated distribution of the mean number of signals per cell in tissue sections allowed finding a cut off value in the total number of signals per cell differentiating between tumor and control tissue cell populations with defined liabilities. The sensitivity and specificity of this method was evaluated with a receiver operating characteristic curve (ROC).

4 Results

4.1 Nucleic acid extraction

4.1.1 Extraction of genomic DNA

With the help of the Gentra Puregene® kit (Gentra-Systems, Minesota, USA) it was possible to obtain good quality DNA from whole feline blood as well as from feline kidney tissue. The concentration of the isolated DNA is shown in the table 10.

Table 10: Concentration of the isolated DNA with the Gentra Puregene® kit (Gentra-Systems, Minesota, USA)

Species	DNA sample No.	Breed	Tissue	Concentration ng/µl
Felis catus	2981	European short hair	Whole blood	147
Felis catus	2985	Persian	Kidney	515

From the gained DNA aliquots were made and stored at - 20°C until use.

4.2 Primer test

The efficiency of the designed primers for the FA-SAT DNA was tested by a conventional PCR in a Gene Amp® PCR System 2400 (Perkin Elmer). Several PCR reactions were performed with different annealing temperatures in order to find the ideal reaction conditions. The optimal reaction conditions for the selected primers are shown in table 3.

The analysis of the PCR product was performed using a 2 % TBE gel electrophoresis as shown in figure 3. The expected band of around 500 base pairs was observed.

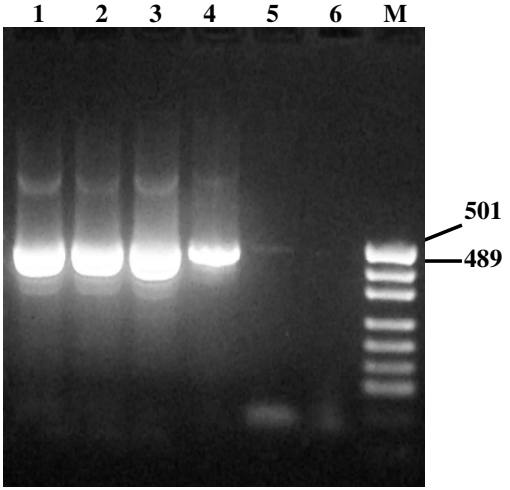


Figure 3: 2 % gel electrophoresis in which the PCR products of the primer test are shown. The expected band of around 500 base pairs was observed. 1: DNA number 2981 (1.5 ng); 2: DNA number 2981 (0.15 ng); 3: DNA number 2985 (5.0 ng); 4: DNA number 2985 (0.5 ng); 5: DEPC-H_2O; 6: DEPC-H_2O; M: pUC19 DNA/MspI (HpaII) Marker 23 ready to use (MBI Fermentas, St. Leon-Rot).

4.3 Cloning of the major feline satellite DNA

The cloning of the major satellite DNA (FA-SAT) was performed using the AccepTor™ Vector Kit (Novagen, Merk, Darmstadt). Of the multiple bacterial colonies obtained, a random selection was performed for verification of the desired insert (4.4).

4.4 Verification of the major feline satellite DNA family FA-SAT insert in the E. coli strain „NovaBlue Giga Singles™ competent cells" in combination with the „pST-Blue-1" vector

The used system (Acceptor™ Vector Kit, Novagen, Merk, Darmstadt) provides a blue/white phenotype that facilitates the screening of the recombinants. The inserted fragment prevents the production of α-peptide, resulting in a white colony phenotype.
Some of the obtained white colonies (with the expected insert) as well as blue colonies (as negative control without insert) were selected for verification of the DNA insert.

4.4.1 Analysis of the obtained insert

The selected white and blue colonies were cultivated again in fluid culture media. Of each obtained culture 0.5 µl mixed with 9.5 µl DEPC-H_2O were denatured under high temperature conditions (95 °C).

Using specific oligonucleotide primers (3.3.2.1) derived form the major feline satellite DNA family FA-SAT reported by Fanning (1987) with the Gene Bank data base accession number X06372, a PCR reaction was performed. An expected DNA band of approximately 500 base pairs was observed in the selected white colonies, but not in the blue colony in a 2 % gel electrophoresis. The obtained DNA fragments are show in figure 4.

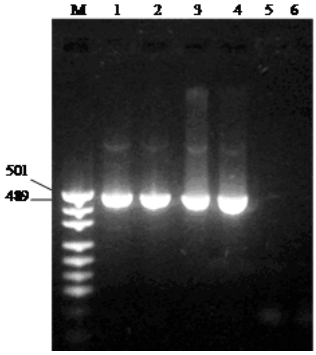


Figure 4: 2 % gel electrophoresis of the PCR reaction with specific oligonucleotide primers for the FA-SAT DNA on bacterial colonies. M: pUC19 DNA/MspI (HpaII) Marker 23 ready to use (MBI Fermentas, St. Leon-Rot); 1: white colony; 2: white colony; 3: white colony; 4: white colony; 5: blue colony; 6: DEPC-H_2O

4.5 Sequencing of the obtained insert

Two of the colonies which showed the expected band near the 480-500 base pair marker band were cultivated again in fluid culture media. From these cultures the plasmid was isolated using the NucleoSpin® Plasmid kit (Marcherey-Nagel, Düren).

DNA sequencing of the selected clones named „PAA 16" and „PAA 7" was performed by MWG Biotech GmbH, Ebersberg.

In order to classify the obtained DNA sequence BLAST analyses were performed using the nucleotide data base of the National Center for Biotechnology Information (NCBI). A homology of 95 % for both cloned inserts with the major satellite DNA family (FA-SAT) in the domestic cat (Gene Bank data base accession number X06372) was found. One of the clones (PAA 16) was selected for further experiments.

The analyzed sequence of the clone PAA 16 is shown in figure 5.

1	CACCGTCCCT	TGTGCCCTCA	ACTCATCCCT	GCAGGGGATC	ACTGCCCTGC
51	TGTGGGTTCA	ACGCCTGGAG	CCTCCTGGGA	CCTTACCCTA	ACCGTAACCT
101	AACCCCTAAC	CGGGACCCTC	CCCCGAAACC	TAAGCCTGCC	TCGATCCCTA
161	ATTCTGCATT	TGGGCCGCCT	TCTGGCATGG	GCCCTCACTT	CAGCCCTCGA
201	GGGAGATTAC	GAGACCGGAG	TCGCTTCGGC	CCTGGCGACG	TCCCTGGCCC
261	GAACCCTAAC	CCTAGCTAAG	GCTCTCCCCT	CATGGGGACC	CAATTCCAAC
301	GTCTGACAGC	ATCACGTGCC	TGGGGTCCAG	AAGCTGTGCA	GGCTGACTCA
361	GCAAGGCACC	TGGAAGCTCA	CCATGTCGTT	TCCCAGGCCC	GGCTGCAGCT
401	CCCGAGAGGA	TTGCGGACGC	CTCCTCGACC	GCGGAAGGAA	CAACATGT

Figure 5: Inserted DNA sequence of the PAA 16 clone with 95 % homology to published satellite DNA family (FA-SAT) in the domestic cat

4.6 Efficiency of probe labeling

After linearization of the double strand DNA with the restriction enzyme Eco57MI (MBI Fermentas, St. Leon-Rot) and purification of the DNA, the probe was labeled with digoxigenin. The labeling method allows synthesizing up to 10 µg of labeled probe starting from 1 µg of purified DNA.

The labeling was tested by direct detection via spot test, the concentration of the product was approximately 100 ng/µl.

4.7 Fluorescence in situ hybridization of the feline FA-SAT DNA

FISH experiments were first designed for the in vitro study of five permanent cell lines. After the statistical analysis of the in vitro study, the same method was designed for the paraffin embedded tissue sections.

4.7.1 FISH in cell lines

For the analysis of the hybridization signals digital images were taken from 200 cells per cell line and analyzed with Adobe Photoshop CS2 and UTHSCA ImageTool 3.0 (University of Texas Health Science Center, San Antonio, Texas). For the signal scoring, size and number of the signals per cell were evaluated. The signals had a size ranging from 0.5 µm to 4.8 µm, all signals with a diameter ≥ 2 µm were defined as large signals, and all those with a diameter below 2 µm were defined as small signals. An example of large and small signals is shown in figure 6.

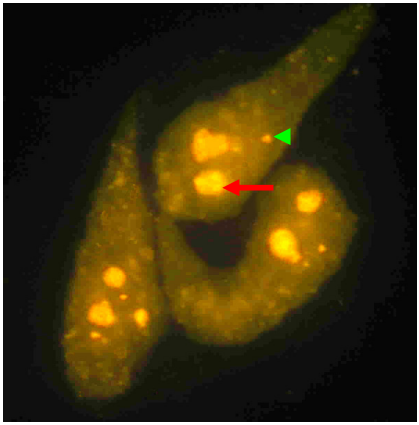


Figure 6: Control cell line in which large signals (red arrow) and small signals (green arrowhead) can be appreciated, Obj. x 100.

4.7.1.1 Analysis of the large signals

For the fluorescence in situ hybridization of the fibrosarcoma cell line FS I the analysis of the large signals showed cells without any signal as well as cells with up to 3 signals per cell as maximum. The median value for large signals in this cell line was zero. An overview of the quantitative distribution of the number of large signals per tumor cell in this cell line is shown in figure 7.

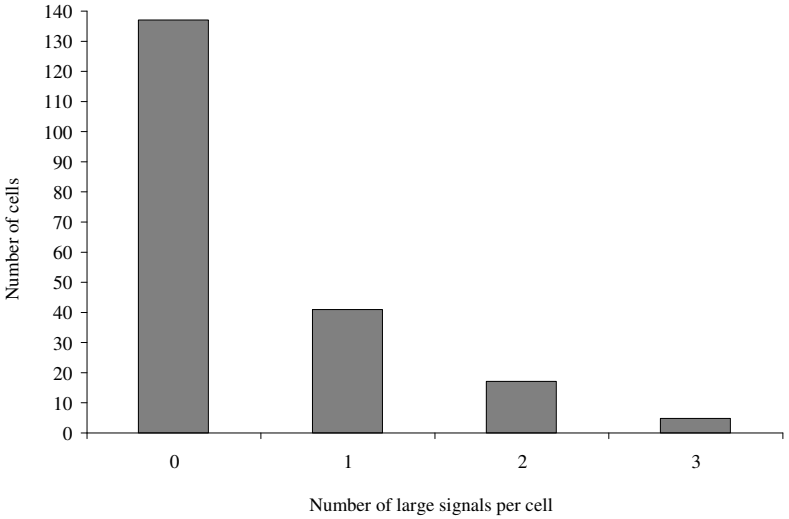


Figure 7: **Quantitative** distribution of the number of large signals per tumor cell in the feline fibrosarcoma cell line FS I (total number of cells: n= 200)

The analysis of the fibrosarcoma cell line FS II showed cells without any large signal as well as cells with 3 signals per cell as maximum. The median value for large signals in the FS II was zero. An overview of the quantitative distribution of the number of large signals per tumor cell in this cell line is shown in figure 8.

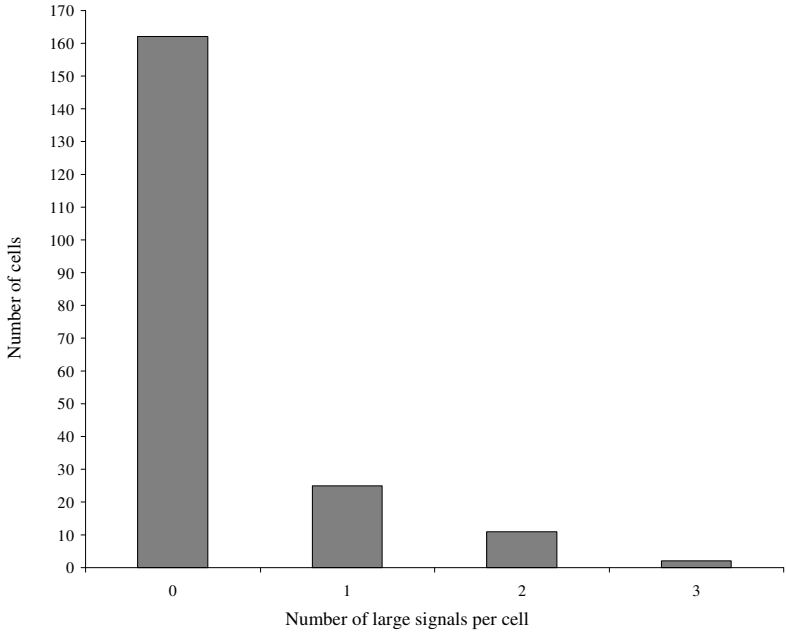


Figure 8: **Quantitative** distribution of the number of large signals per tumor cell in the feline fibrosarcoma cell line FS II (total number of cells: n= 200)

In the fibrosarcoma cell line FS III there were cells without any large signal as well as cells with up to 4 signals per tumor cell as maximum. The median value was zero signals per cell. An overview of the quantitative distribution of the number of large signals per tumor cell in this cell line is shown in figure 9.

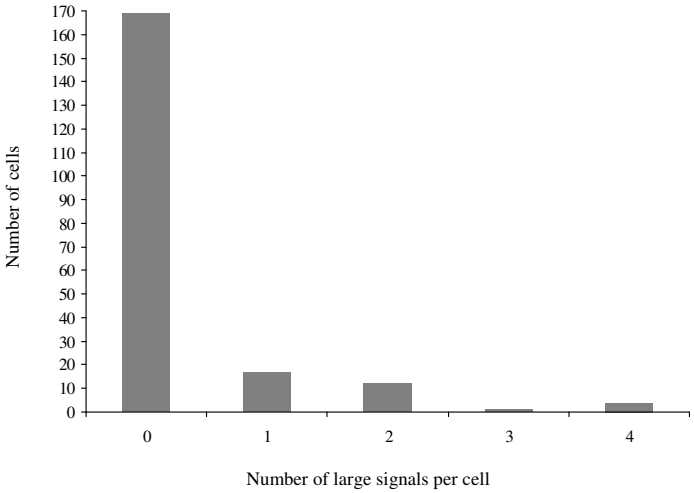


Figure 9: Quantitative distribution of the number of large signals per tumor cell in the feline fibrosarcoma cell line FS III (total number of cells: n= 200)

In the fibrosarcoma cell line FS IV there were cells without any large signal as well as cells with up to 3 signals per tumor cell as maximum. The median value of large signals was zero. An overview of the quantitative distribution of the number of large signals per tumor cell in this cell line is shown in figure 10.

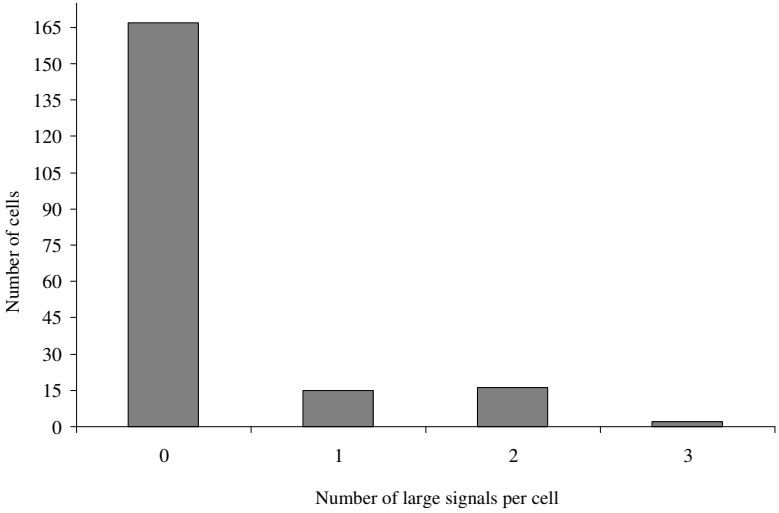


Figure 10: Quantitative distribution of the number of large signals per tumor cell in the feline fibrosarcoma cell line FS IV (total number of cells: n= 200)

The fibroblastic cell line derived from normal feline skin which was used as control had cells with one large signal per cell as minimum as well as cells with up to 5 large signals per cell as maximum. The median value was three large signals per cell. An overview of the quantitative distribution of the number of large signals per normal skin fibroblast is shown in figure 11.

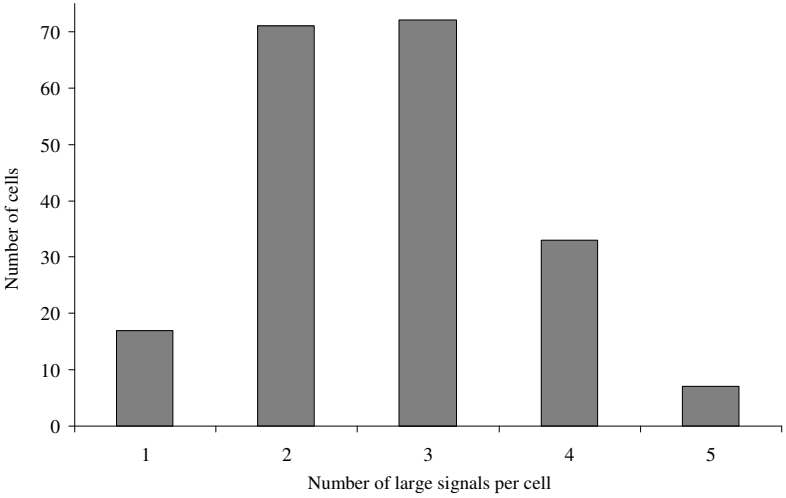


Figure 11: Quantitative distribution of the number of large signals per cell in the control cell line (total number of cells: n= 200)

4.7.1.1.2 Comparison of the FISH results for large signals in cell lines

The statistical analysis of the mean number of large signals per cell of the control cell line and of all fibrosarcoma cell lines put together (FS I, FS II, FS III, and FS IV) showed a statistical significant difference ($p < 0.0001$) with a Post-hoc-test (Nemenyi). No statistical significant difference for large signals was found with a Post-Hoc test (Nemenyi) between the different feline fibrosarcoma cell lines (FS I-FS IV).

The results of the FISH analysis for large signals for all cell lines are summarized in figure 12 as Box-and-Whisker-Plots.

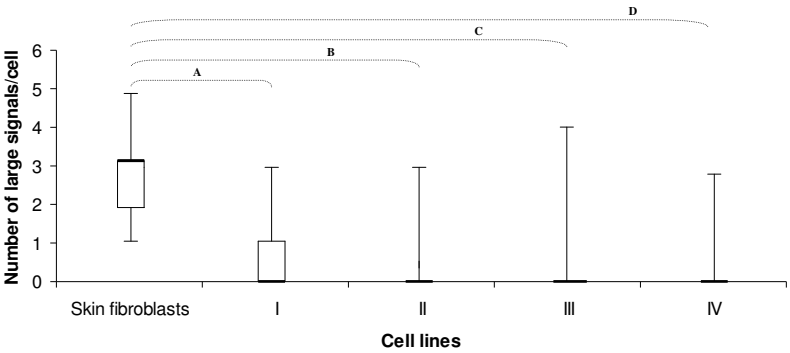


Figure 12: Box-and-Whisker-Plots of the total number of large signals per cell of the studied cell lines. I: Feline fibrosarcoma I; II: Feline fibrosarcoma II; III: Feline fibrosarcoma III; IV: Feline fibrosarcoma IV A: $p < 0.0001$; B: $p < 0.0001$;
C: $p < 0.0001$; D: $p < 0.0001$ (total number of cells: n= 200)

4.7.1.2 Analysis of the small signals

The analysis of the small FISH signals in the fibrosarcoma cell line FS I showed a minimum of 5 signals per cell, and a maximum of 36 signals per cell. The median value for small signals was 16 signals per cell. An overview of the quantitative distribution of the number of small signals per tumor cell in this cell line is shown in figure 13.

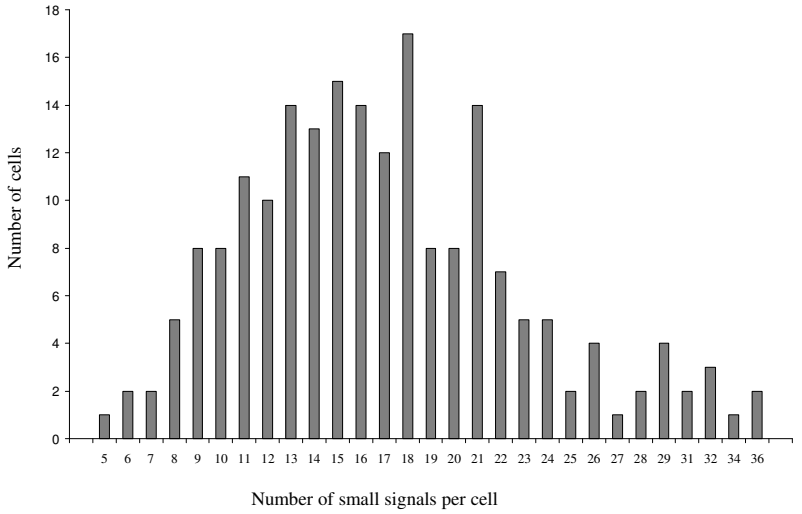


Figure 13: Quantitative distribution of the number of small signals per tumor cell in the feline fibrosarcoma cell line FS I (total number of cells: n= 200)

The fibrosarcoma cell line FS II showed cells with 4 small signals per cell as minimum, and a maximum of 38 small signals per cell. The median value was 18 small signals per cell. An overview of the quantitative distribution of the number of small signals per tumor cell in this cell line is shown in figure 14.

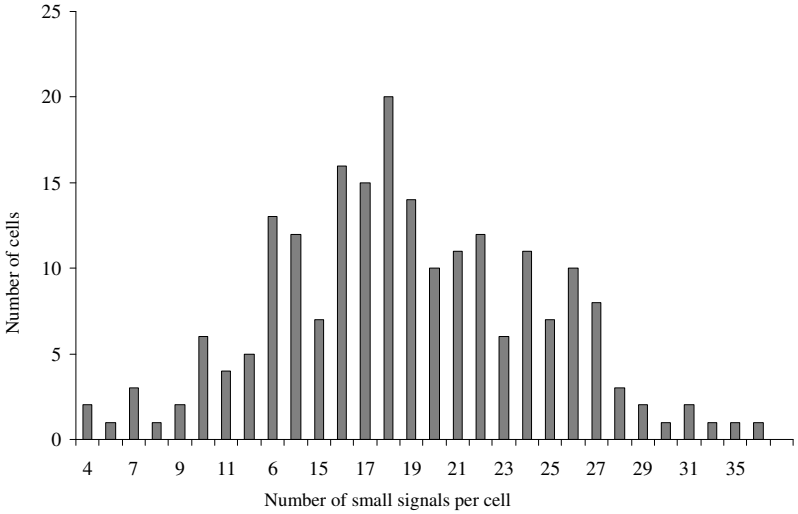


Figure 14: Quantitative distribution of the number of small signals per tumor cell in the feline fibrosarcoma cell line FS II (total number of cells: n= 200)

In the fibrosarcoma cell line FS III 5 small signals per cell were observed as minimum as well as up to 45 small signals per tumor cell as maximum. The median value for small signals was 21 signals per cell. In figure 15 an overview of the quantitative distribution of the number of small signals per tumor cell in this cell line is shown.

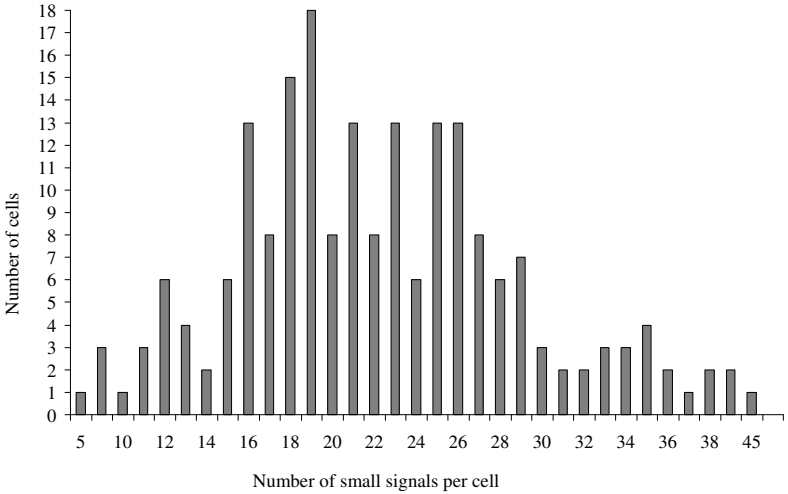


Figure 15: Quantitative distribution of the number of small signals per tumor cell in the feline fibrosarcoma cell line FS III (total number of cells: n= 200)

A minimum of 10 small signals per tumor cell were observed in the fibrosarcoma cell line FS IV, as well as 39 small signals per tumor cell as maximum. The median value observed for small signals in this cell line was of 24 signals per tumor cell. An overview of the quantitative distribution of the number of small signals per tumor cell in this cell line is shown in figure 16.

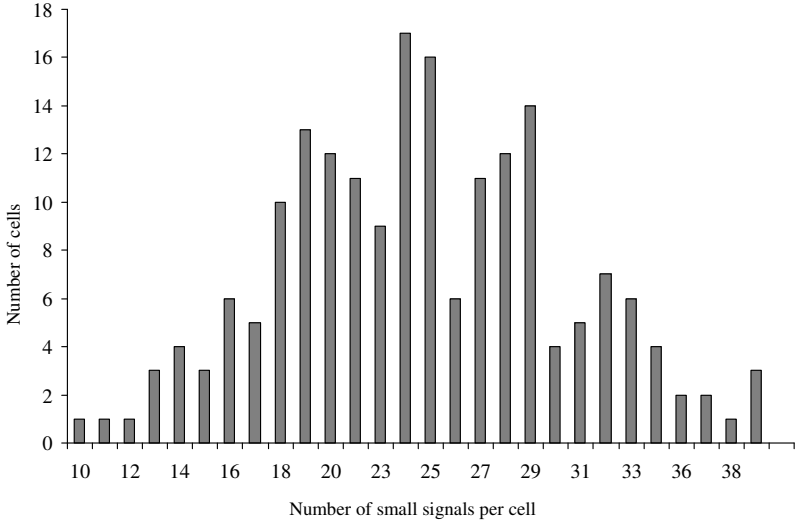


Figure 16: Quantitative distribution of the number of small signals per tumor cell in the feline fibrosarcoma cell line FS VI (total number of cells: n= 200)

The fibroblastic cell line derived from normal feline skin which was used as control had cells with 1 small signal per cell as minimum as well as small 15 signals per cell as maximum. The median value for small signals in this cell line was 4 signals per cell. An overview of the quantitative distribution of the number of small signals per normal skin fibroblast is shown in figure 17.

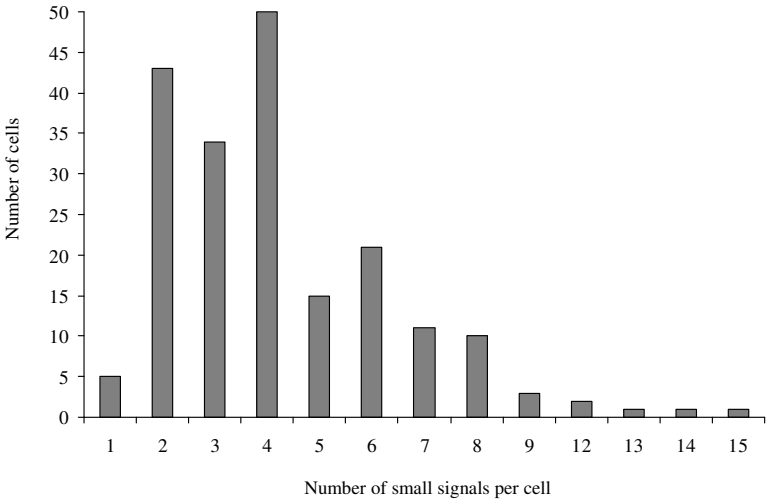


Figure 17: Quantitative distribution of the number of small signals per cell in normal feline skin fibroblasts in vitro (total number of cells: n= 200)

4.7.1.2.1 Comparison of the FISH results for small signals in the different cell lines

For statistical analysis of the small signals a Post-hoc-test was performed. A statistically significant difference ($p < 0.0001$, Post-hoc-test, Nemenyi) was found between the mean number of small signals per cell in the control cell line and all fibrosarcoma cell lines put together (FS I, FS II, FS III, FS IV). The correlation between the different fibrosarcoma cell lines with a Post-hoc-test, Nemenyi showed statistical significant differences between some of them. The differences between these groups are shown in the table 11.

Table 11: Statistically significant differences between the mean number of small signals per cell in different feline fibrosarcoma cell lines found with a Post-hoc-test (Nemenyi)

Difference between feline fibrosarcoma cell lines	***p value***
FS I – FS III	< 0.0001
FS I – FS IV	< 0.0001
FS II – FS IV	< 0.0001
FS II – FS III	< 0.01

The results of the FISH analysis for small signals for all cell lines are summarized in figure 18 as Box-and-Whisker-Plots test.

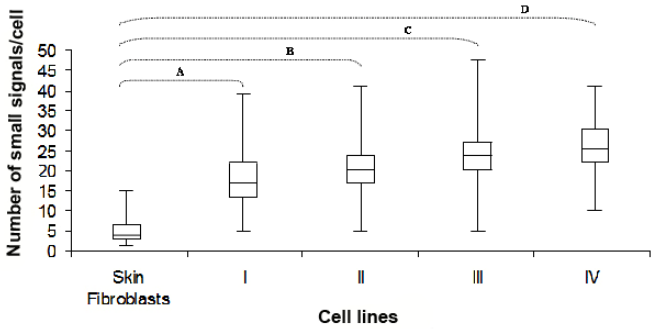


Figure 18: Box-and-Whisker-Plots of the total number of small signals per cell of the studied cell lines. I: Feline fibrosarcoma I; II: Feline fibrosarcoma II; III: Feline fibrosarcoma III; IV: Feline fibrosarcoma IV A: $p < 0.0001$; B: $p < 0.0001$; C: $p < 0.0001$; D: $p < 0.0001$ (total number of cells: n= 200)

In figure 19, the hybridization pattern of the control cell line and the feline fibrosarcoma cell lines is depicted.

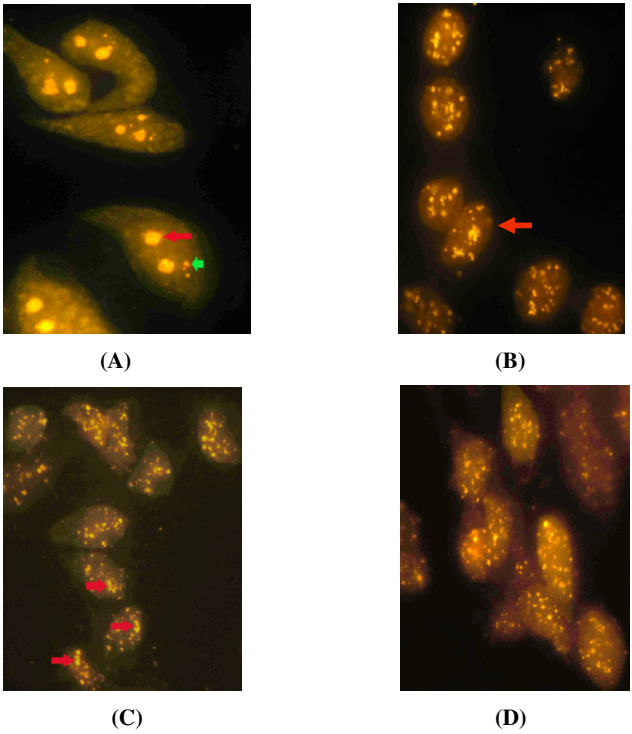


Figure 19: Hybridization pattern of the feline major satellite DNA (FA-SAT) in cell lines:

(A) Feline fibroblastic cell line from normal skin used as a control. The hybridization pattern is composed mainly of large signals (large red arrow) and few small signals (small green arrow), Obj. x 100.

(B) Feline fibrosarcoma cell line I. Most of the cells observed present a hybridization pattern composed only of small signals. One cell (arrow) presents both large and small signals, Obj. x 100.

(C) Feline fibrosarcoma cell line III. In most of the cells the hybridization pattern is composed only of small signals and in some cells large signals (arrows) are observed additionally, Obj. x 100 .

(D) Feline fibrosarcoma cell line IV. All cells observed present a hybridization pattern composed only of small signals, Obj. x 100.

4.7.2 FISH on paraffin-embedded tissue sections

The selected tissues consisted of 30 biopsies from spontaneous feline fibrosarcomas from 28 cats. For each case two tissues were studied. One of them was tumor tissue and the other was non affected skin of the same patient. For each sample 100 tumor cells and 100 dermal fibroblasts from non-affected skin were analyzed. For each cell population digital images were taken until the indicated number of cells was analyzed and analyzed with Adobe Photoshop CS2 and UTHSCSA ImageTool 3.0 (University of Texas Health Science Center, San Antonio, Texas).

The FA-SAT signals in tissue sections presented only slight variations in their size ranging from 0.5 µm to 1.7 µm in diameter, compared with those in cell lines (0.5 µm to 4.8 µm). Therefore, no differences were made regarding signal size and only the total number of signals per cell was evaluated for the signal scoring.

The mean value of the total number of signals per cell in the tumors and in the dermal fibroblasts of non-affected skin of the same patient were analyzed with the Wilcoxon-Mann-Whitney-Test. The hybridization pattern observed in tumor cells differed significantly ($p < 0.0001$, Wilcoxon-Mann-Whitney-Test) in the mean number of signals per cell (9.6) from that in dermal fibroblasts (7.6). The results of the statistical analysis are summarized in figure 20 as Box-and-Whisker-Plot.

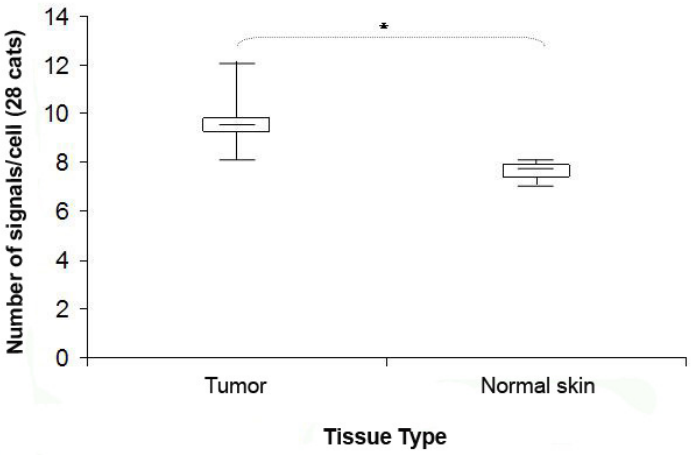
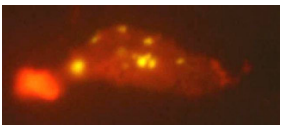
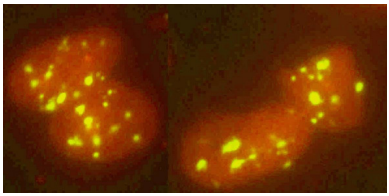


Figure 20: Box-and-Whisker-Plot of the total number of signals per cell in tumor tissue and dermal fibroblasts of normal skin of 28 patients; * $p < 0.0001$ (Wilcoxon-Mann-Whitney-Test)

In figure 21, the hybridization pattern of dermal fibroblasts of non-affected skin and tumor fibroblasts are shown.



(A)



(B)

Figure 21: Hybridization pattern observed in tissue sections of fibroblasts of non-affected skin (A), and feline fibrosarcoma tumor cells (B). The number of signals observed in the tumor cells is higher than that of non-affected fibroblasts, Obj. x 100.

4.7.2.1 Feline fibrosarcomas which were recurrent

In two of the patients the tumors recurred after some time. For the cat with the case number T 6035/04, a significant statistical difference $p < 0.0001$ (two factorial analysis of variance) between the mean number of signals per tumor cell and per skin fibroblast of non-affected skin in the primary tumor was found.

For this patient (T 6035/04) a recurrence of the tumor was analyzed later (T 8904/04). A significant difference $p < 0.0001$ (two factorial analysis of variance) between the mean number of signals per tumor cell and per dermal fibroblast of non-affected skin in the recurrent tumor was found, too. In this patient no significant differences in the mean number of signals per cell were found neither for tumor cells of the first tumor (T6035/04) and its recurrence (T8904/04), nor between normal dermal skin fibroblasts of the first biopsy and the second one. The comparison of the first tumor with its recurrence and their correspondent skin biopsies in this patient are presented in figure 22 as Box-and-Whisker-Plots.

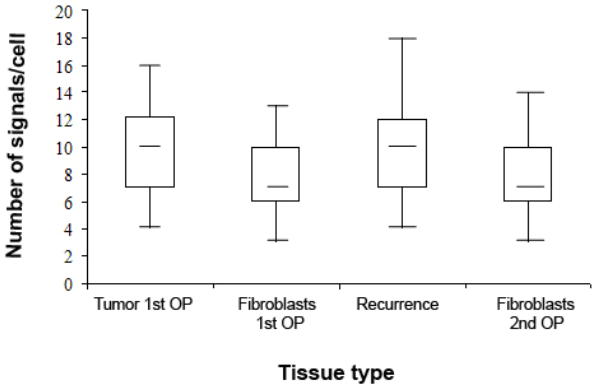


Figure 22: Box-and-Whisker-Plot of the number of signals per cell of tumor cells of the first tumor (T6035/04) and its recurrence (T8904/04) as well as their correspondent skin biopsies

A second cat with recurrence of the tumor was analyzed, too. For this patient (T 6125/05) a statistical significant difference $p < 0.0001$ (two factorial analysis of variance) between the mean number of signals per tumor cell and per dermal fibroblast of non-affected skin was found in the primary tumor. Also the statistical analysis of the recurrence (T8535/06) showed a significant difference $p < 0.0001$ (two factorial analysis of variance) between the mean number of signals per tumor cell and per dermal fibroblast of non-affected skin. For this patient no significant differences in the mean number of signals per cell were found neither for tumor cells of the first tumor (T6125/05) and its recurrence (T8535/06), nor between normal dermal fibroblasts of the first biopsy and the second one. The comparison of the first tumor with its recurrence and their corresponding skin biopsies in this patient is presented in figure 23 as Box-and-Whisker-Plots.

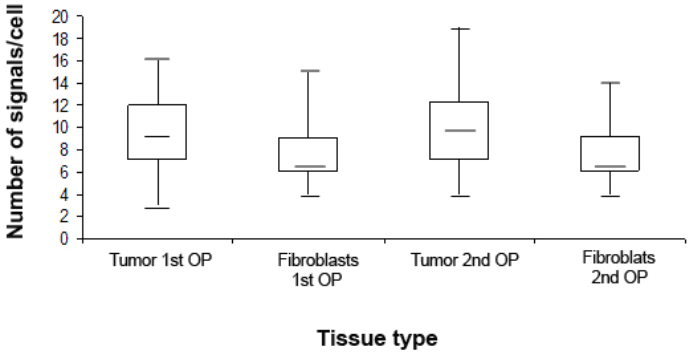


Figure 23: Box-and-Whisker-Plot of the number of signals per cell of tumor cells of the first tumor (T6125/05) and its recurrence (T8535/06) as well as of their correspondent skin biopsies

4.7.3 Statistical analysis of the estimated distribution of the mean number of signals per cell in tumor tissue and non-affected skin of 28 cats

This statistical model uses the mean number of signals per cell of each tissue (tumor and non-affected fibroblasts) within each cat, assumed to be nearly normally distributed between the cats. From this data the mean and standard deviation were calculated over all the cats to estimate the normal distribution parameters of tumor cells as well as non-affected fibroblasts, all based on data of our 28 studied patients. So, it was possible to analyze the estimated normal distribution of tumor and non-affected cell populations in order to find an acceptable cut off value where both cell populations were separated.
The model was applied with three different numbers of cell samples (100, 50, and 25 cells per cat), and the results are shown in figures 24, 25, and 26.

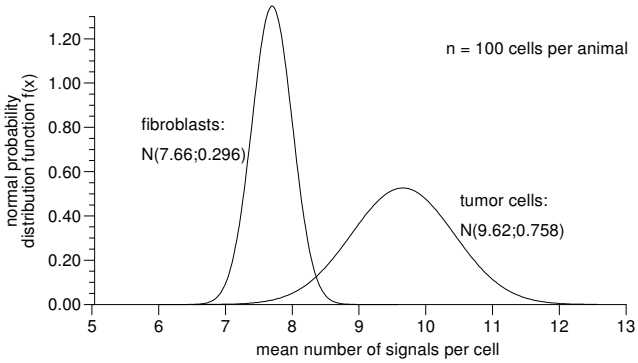


Figure 24: Estimated normal distributions of the mean numbers of signals per cell in tumor tissue and fibroblasts from non-affected skin with a sample size of 100 cells (tumor and non-affected fibroblasts) taken from 28 cats. N: normal distribution (mean; standard deviation); n = number of cells examined per animal and sample

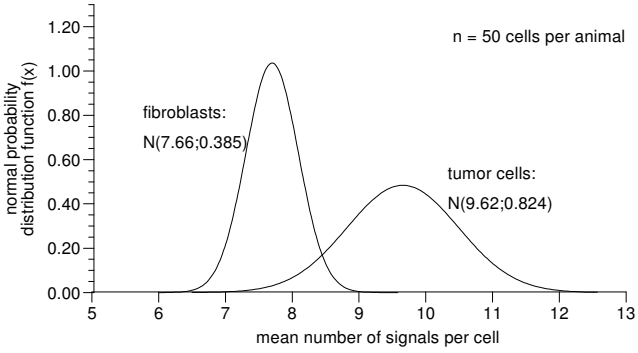


Figure 25: Estimated normal distributions of the mean numbers of signals per cell in tumor tissue and fibroblasts from non-affected skin with a sample size of 50 cells (tumor and non-affected fibroblasts) taken from 28 cats. N: normal distribution (mean; standard deviation); n = number of cells examined per animal sample

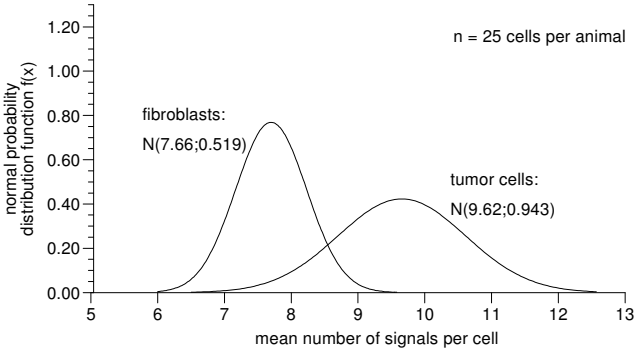


Figure 26: Estimated normal distributions of the mean numbers of signals per cell in tumor tissue and fibroblasts from non-affected skin with a sample size of 25 cells (tumor and non-affected fibroblasts) taken from 28 cats. N: normal distribution (mean; standard deviation); n = number of cells examined per animal and sample

Additionally, a receiver operating characteristic curve (ROC) was established for each estimated normal distribution (n=100, n=50, n=25) in order to establish their sensitivity and specificity. The estimated normal distribution of mean numbers of signals per cell and its correspondent ROC-plot allowed to evaluate the methodology performed in this work and its possible diagnostic value.

The cut off for all three cell samples sizes (n=100, n=50, n=25) was designed as 8.3 signals per cell. Therefore, this is the middle point (cut off) at which both studied cell populations (neoplastic and non-neoplastic) are separated. All cells with a signal count under 8.3 were considered non-neoplastic and above 8.3 classified as tumor cells. Assuming the normal distribution model for a cell sample of 100 cells the ROC-Plot showed a sensitivity of 96 % with 98 % specificity; for a cell sample of 50 cells the sensitivity was 94 % with 95 % specificity; and for a cell sample of 25 cells the sensitivity was 91 % with 90 % specificity. The corresponding statistical analysis of the areas under the curve (AUC) for the ROC-Plot were AUC: 0.965 (n=25), AUC: 0.984

(n=50), and AUC: 0.992 (n=100). The AUC is defined as an estimation of the proportion for correct differentiation between tumor and fibroblast cells in this statistical model.

In figure 27 the ROC-Plot for the three cell samples (n=100, n=50, n=25) are shown.

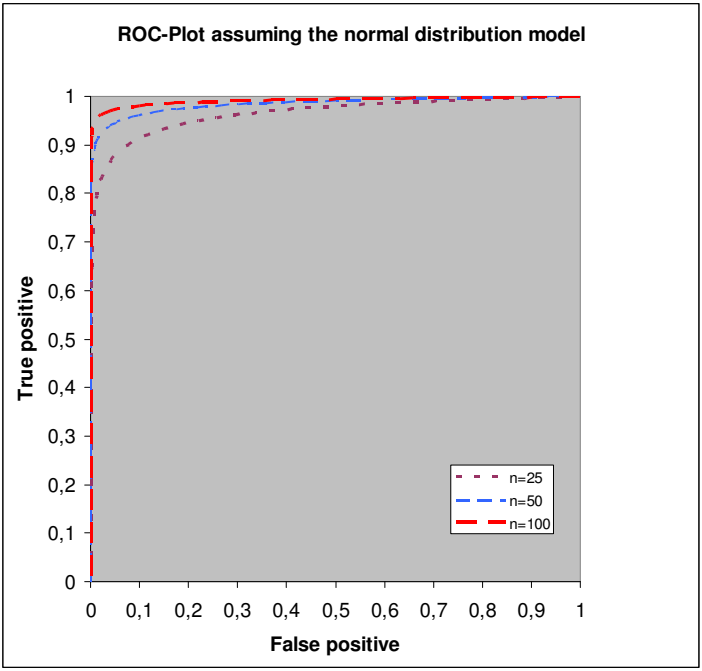


Figure 27: ROC-Plot assuming the normal distribution model for three cell sample sizes (n=100, n=50, n=25). n: number of cells evaluated per animal and sample

5. Discussion

In spite of the importance of fibrosarcomas in cats, the pathogenesis of this tumor has not been completely elucidated. In this work we focused on the study of the major feline satellite DNA FA-SAT in vitro and in fibrosarcoma biopsy samples. We cloned the reported FA-SAT sequence and our results showed 95 % homology with the original sequence reported first by Fanning (1987).

Santos et al. (2004) described the chromosomal localization of this sequence in the domestic cat. The authors reported the FA-SAT DNA to be present in some telomeres and centromeres of 22 of the 38 feline chromosomes. Here we studied the hybridization pattern of the FA-SAT in tumor and non-affected dermal fibroblast, in order to detect possible differences existing between these two cell populations.

A possible role of the FA-SAT in feline fibrosarcoma pathogenesis has only been previously discussed by Santos et al. (2006) in one occasion. His report is based on the findings of a single fibrosarcoma case through the study of the FA-SAT on chromosomes.

In this work, we report the study of the FA-SAT by fluorescence in situ hybridization in five cells lines and 30 biopsy cases.

5.1 Analysis of differences in the hybridization pattern of the FA-SAT in cell lines and tissue sections

The fluorescence in situ hybridization of the FA-SAT in the feline fibrosarcoma cell lines showed a hybridization pattern different from the one observed in the cell line of dermal fibroblasts from non-affected skin used as a control. Differences between the hybridization pattern of paraffin-embedded tumor tissues and skin biopsies were also observed.

In cell lines, the hybridization pattern of dermal fibroblasts showed two different types of signals throughout the multiple hybridization experiments and passages. All normal dermal fibroblasts presented always signals larger than 2 µm which were classified as large signals, and at the same time presented at least one signal with a diameter below 2 µm which was then classified as small signal. The number of large and small signals showed some variations between cells. For 88.5 % of the control cell population there

were two to four small signals per cell, and all cells (100 %) presented always one or more large signals with 71.5 % of the cell population ranging between two and three signals per cell. On the other hand, for all fibrosarcoma cell lines more than 90 % of the cells presented more than 10 small signals per cell. The FS I had 91 % of the cells with more than 10 small signals per cell, the FS II 95.5 %, the FS III 98 %, and in the FS IV 100 %. All fibrosarcoma cell lines presented populations with more than 90 % of the cells having none or only one large signal per cell, with the exception of the FS I with 89 %. For the other fibrosarcoma cell lines this finding was: FS II 93.5 %, FS III, 93 %, and FS IV 91 %.

Although most of the control and feline fibrosarcoma cell populations had a narrow range of their numbers of signals, there were some cells which presented some larger variations. These differences might be related to the fact that in a population of cells it is possible to find some of the cells in different stages of the replication cycle. Therefore, some of the cells will have a higher amount of nuclear content due to the replication of the nuclear components so that the number of signals in the cells will show some variation through the replication cycle. In addition to that, some intrinsic factors in the hybridization methodology like the individual cell hybridization efficiency might possibly influence at least to some degree the number of observed signals. Nevertheless, to the best of our knowledge, although this last aspect could not be ignored, we do not expect that it has influenced our results in a significant way.

The differences in the FISH signal size in paraffin-embedded fibrosarcomas as well as in skin biopsies were not so obvious when compared to the ones in the cell lines (0.5 µm to 4.8 µm). For the tissue samples, the signals size ranged from 0.5 µm to 1.7 µm. Therefore, for the statistical analysis of the paraffin-embedded material just the total number of signals per cell in both populations (control and tumor) was evaluated and no differences were made as to their size. These differences in the size of the signals between tissue sections and in cultured cells could be related with the number of repeats of the major satellite DNA in telomeres and centromeres. As mentioned in the literature overview (2.4), the major satellite DNA is located in telomeres and centromeres of some feline chromosomes as a repetitive sequence. The number of times that this repetitive DNA is present in those locations is not always equal, and could present variations from one chromosome to the other, like the human alphoid DNA which varies in amount from chromosome to chromosome forming larger unit repeats of

approximately 300-5000 kb (Huntington, 1990). The larger the number of satellite repetitions in those sites, the larger the signal size will be.

In addition to that, Santos et al. (2004) reported the chromosomal localization of the major satellite DNA in the domestic cat. In his work a polymorphism of the major satellite DNA was found in all studied cats between homologous chromosome pairs of some of the chromosomes. Thereby, the size of the hybridization signals of the FA-SAT might present some variations within single cells and between different cells of the same population.

A statistical significant difference in the total number of small signals $p < 0.0001$ (4.7.1.2.1) and large signals $p < 0.0001$ (4.7.1.1.2) was found in vitro when we compared the control cell line with all fibrosarcoma cell lines.

In paraffin-embedded material, a significant statistical difference $p < 0.0001$ (4.7.2) was found between the total number of signals in tumor cells and fibroblasts of non-affected skin. The tumor cells showed a mean value of 9.6 signals per cell and a median of 9.4, while for normal skin fibroblasts it was a mean value of 7.6 signals per cell and a median of 7.7. Some slight variations in the number of signals in tissue sections were expected to be present between cells, in part because of the effect of the normal replication cell cycle on the duplication of the nuclear material in form of an increase in the number of signals and, in addition to that, the number of signals can present some variations in tissue sections because through the sectioning of the tissue some amount of nuclear material might have been cut out. Therefore, it was to be expected that the amount of nuclear content is visible with slight variations between cells within the same tissue. Our interpretation of this aspect was that cells in which more nuclear material was cut out, presented a lower number of signals than those in which most of the nuclear content was retained.

In this work, the in vitro experiments were used as possible model for what could be expected in vivo. These experiments showed a significant difference in the total number of signals between the control cell line and the fibrosarcoma cell lines.

The cell lines used in this work were previously established and characterized by Löhberg-Grüne in her doctoral thesis (annex 8.1). Löhberg-Grüne (2009) showed that all fibrosarcoma cell lines had an abnormal karyotype, and that all fibrosarcoma cell lines had in most of their cells a higher number of chromosomes than the normal 38 chromosomes observed in the control cell line. The fibrosarcoma cell lines had up to 144 chromosomes per cell in one of the cell lines (FS IV) (annex 8.3). Wasieri (2009)

through cloning of Löhberg-Grüne's cell lines showed that all cloned fibrosarcoma cell lines had a higher than normal and still varying number of chromosomes as well as a centrosome hyperamplification.

Regarding our control cell line, we interpret the results of the in vitro dermal fibroblasts as the mean number of signals in a cell population with normal diploid feline karyotype (2n=38) so that the number of signals in this non-affected population of cells could be used as normal reference for the study of fibrosarcomas in vitro. Likewise, biopsies of non-affected skin should represent a normal diploid cell population. Therefore, we used it as the normal reference for the study of the fibrosarcoma tissue.

The increased number of chromosomes observed by Löhberg-Grüne (2009) in the fibrosarcoma cell lines used in this work can explain in part the higher number of signals observed when compared with the control cell line. For this reason, we can assume that in those cells in which a higher number of signals was observed a higher number of chromosomes is likely. As mentioned in the literature review (2.7), chromosomal numerical aberrations are also known by studies of other authors to occur in feline fibrosarcomas in vitro. Therefore, it is to be expected that numerical abnormalities will also be present in fibrosarcomas in vivo.

Biopsy samples should represent the in vivo characteristics of the studied tissue. In all our fibrosarcoma biopsies the mean number of signals was always higher than the mean number of signals in control tissue. Based on our in vitro results we hypothesize that the increase in the mean number of signals in tumor cells could represent also a higher number of chromosomes in neoplastic tissue when compared with the control skin tissue. Our results in vitro and in vivo corroborate observations made by other authors in mammalian cells, human mesenchymal tumors, and feline fibrosarcoma (Heartlein et al., 1988; Haaf et al., 1992; Gisselsson et al., 1999; Santos et al., 2006).

Repetitive DNA sequences have been implicated in playing a role in DNA rearrangement in mammalian cells. Heartlein et al. (1988) reported an approximately 50 % increase in chromosome number and marked changes in chromosome structure in Chinese hamster ovary cells transfected with human alphoid satellite DNA. Additionally, it was reported that aberrant chromosomes had more alphoid DNA, suggesting that it may serve as hot spot for rearrangements. The chromosomal instability observed in the transfected cells suggests that alphoid satellite DNA organization could have a functional role in the maintenance of chromosome stability (Heartlein et al., 1988).

Earlier studies have also shown an association between amplified human alphoid DNA, generated by transfection of animal cells, and chromosomal instability. In African green monkey cells, disturbance of normal chromosome segregation was observed for chromosomes containing large arrays of human alpha satellite DNA (Haaf et al., 1992).
Human mesenchymal tumors have been reported to present a variable stability of chromosomes containing amplified satellite sequences. Chromosomal instability was reported in a human atypical lipomatous tumor which exhibited great intercellular variation in structure and number of chromosomes in association with amplified alpha satellite DNA (Gisselsson et al., 1999).
As discussed in the literature review (2.6), the satellite DNA plays a role in centromere function in normal mammalian cells. Thereby alterations at this specific DNA level could be associated with chromosomal instability which is a common feature of many tumors (Haaf et al., 1992, Gisselsson et al., 1999; Golling, 2005; Michor et al., 2005).
In the study performed by Santos et al. (2006) FA-SAT alterations from one feline fibrosarcoma were reported. In this study, several marker chromosomes with aberrant FA-SAT in situ hybridization signals were found. Besides, chromosomes with amplification of the major satellite DNA were also reported. The FA-SAT DNA sequences were always amplified at the centromere regions of the marker chromosomes. These large blocks of FA-SAT found at the centromere region might suggest that they could be essential for centromere function. Furthermore, the finding of large blocks of satellite DNA sequences in other regions of the marker chromosomes might be the result of chromosomal rearrangements. The amplification of the satellite DNA associated with variable marker chromosomes may be related with chromosomal instability of this tumor (Santos et al., 2006).
The higher number of signals observed in all our tumor material in vitro and in vivo when compared with the control tissues and cells might be associated with an amplification of the major satellite DNA in tumor cells, as well as with chromosomal instability, as postulated also by other authors (Gisselsson et al., 1999; Santos et al., 2006).

5.2 Breed, age, sex, and localization of the tumors

Fibrosarcoma biopsies came mostly from European short hair cats, (23/28), the remaining animals were all of mixed breed (5/28).

No sex predilection was found for the study group, an observation that agrees with the literature (Hendrick et al., 1994a; Goldschmidt and Hendrick, 2002; Shaw et al., 2009).
The mean age of cats in this study was 9 years (range, 4 to 14 years) at the time of biopsy. The age of cats with injection site-associated fibrosarcoma is reported to be lower (with a mean age of 8.6 years) than the age of cats affected by non injection site-associated fibrosarcomas (with a mean age of 10.2 years) (Doddy et al., 1996; AFIP, 2007). The mean age of our cases seems to be slightly over the mean age reported for the injection site-associated fibrosarcomas by some authors.
Most of the analyzed fibrosarcoma biopsy samples (21/28) were tumors located at regions traditionally used by veterinarians for administration of vaccines and other drugs. In our cases the most frequent localizations were the back region including back sides (14/28), and the hind legs (7/28). Although no previous history of injection and/or vaccination at these sites was confirmed, most of our samples were located in anatomical sites previously reported in the literature to be common for the development of injection site-associated fibrosarcomas (Goldschmidt and Hendrick, 2002; Gross et al., 2005; Shaw et al., 2009).

5.3 Fibrosarcomas recurrences

Feline fibrosarcomas are locally invasive and aggressive neoplasms, and complete surgical excision is often difficult. Therefore the incidence of recurrence is usually high (60 % to 75 %) (Gross and Walder, 1992; Briscoe et al., 1998). Of the 28 cats included in this study, just two (7 %) were presented with recurrence of the tumor. For one of the patients (T 6035/04) the time to recurrence (T 8904/04) was about 4 months, while for the second one (T6125/05) the time to recurrence (T8535/06) was 15 months. A recurrence of the tumor between 3 months and 3 years has been reported for the feline injection site-associated fibrosarcomas (Hendrick et al., 1994b; McEntee and Page, 2001).
A statistically significant difference $p < 0.0001$ between the total number of signals in tumor cells and non-affected dermal fibroblasts was observed in the first tumor as well as in the recurrence of both patients (4.7.2.1). No significant statistical differences were observed between the total number of signals in tumor cell populations when comparing the first tumor with its recurrence in both cases. This shows that the recurrent tumor did not present a significant increase in the total number of signals per cell when compared

with the first tumor. These findings suggest that the recurrences of fibrosarcomas in our patients did not represent a special group of tumors with significant differences in their satellite amplification and chromosomal instability.

5.4 Diagnostic relevance of fluorescence in situ hybridization of feline major satellite DNA

The statistical analysis of our results in vitro and in tissue sections showed a significant difference between the total number of signals in tumor cells and fibroblasts. In addition to that, the estimated normal distribution of the mean number of signals per cell and its correspondent ROC curves showed a high sensitivity and specificity (see 4.7.3) for all three cell sample sizes analyzed (n= 100; n= 50; n=25) with over 96.5 % of reliability.
Our results show that this method is useful for the differentiation of tumor cells from normal ones. This is a relevant result, especially when taking into consideration that feline fibrosarcomas are the most common malignant mesenchymal neoplasm of feline skin with a frequent recurrence rate of 60 % to 75 % (Miller, 1991; Gross and Walder, 1992; Hirschberger and Kessler, 2001).

6 Zusammenfassung

Feline Fibrosarkome sind die am häufigsten vorkommenden Hautumoren der Katze. Zwar gibt es verschiedene Hypothesen zur Pathogenese dieser Tumoren, letztendlich ist ihre Entwicklung aber noch in vielerlei Hinsicht nicht geklärt.

Diese Studie untersuchte die "feline major satellite DNA (FA-SAT)" mittels FISH in Fibrosarkomzelllinien, in einer normalen embryonalen Fibroblastenzelllinie und 30 Fibrosarkomen von 28 Katzen.

Von letzteren wurden Biopsien aus dem Tumorgewebe und aus nicht betroffenen gesunden Hautbereichen gewonnen.

Es konnten signifikante Unterschiede im FA-SAT-Hybridisationsmuster zwischen der Fibroblastenzelllinie und den Fibrosarkomzelllinien belegt werden. Ebenso lagen Signalunterschiede zwischen Fibroblasten aus Tumorgewebe und Fibroblasten aus nicht betroffenen Hautarealen im Paraffin-eingebetteten Material vor.

Diese in vitro und ex vivo ermittelten Unterschiede weisen auf eine mögliche Rolle der FA-SAT hinsichtlich chromosomaler Instabilität im felinen Fibrosarkom hin.

Weiterhin konnte diese Arbeit den Nutzen der FISH von FA-SAT in der Differenzierung zwischen Tumorzellen und normalen Zellen in felinen Fibrosarkomen aufzeigen.

6. 1 Summary

Feline fibrosarcomas are the most common skin tumors of cats. Despite this high frequency and the publication of different hypotheses for their pathogenesis by several authors, the alterations accompanying the development of this tumor are still not completely understood.

We studied the feline major satellite DNA (FA-SAT) hybridization pattern by FISH in four fibrosarcoma cell lines and one normal embryonic fibroblastic cell line as well as in 30 fibrosarcomas from 28 cats. Of the latter, tumor tissue biopsies and biopsies of non-affected skin of the same patient were analyzed.

The hybridization pattern of the FA-SAT in the normal fibroblastic cell line differed significantly from that observed in all fibrosarcoma cell lines. The same observation was made for tumor tissues and fibroblasts from non-affected skin in paraffin-embedded material. These differences observed in vitro and in vivo suggest that alterations of the FA-SAT may be associated with chromosomal instability in the feline fibrosarcoma.

Additionally, our FISH results show the usability of the designed method in differentiating tumor cells from normal skin fibroblasts in feline fibrosarcoma.

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7 References

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8 Annexes

Annex 8.1: General details of the patients from which the tumor cell lines came from.

Cell line	Breed	Age in years	Sex	Tumor localization	Tumor development	Boosters or Injections
Skin fibroblasts	ESH	Young fetus	f	--	--	--
FS I	ESH	11	m	Thorax	Not known	Not Known
FS II	ESH	6	mc	Thorax	Fast: in a period of 5 months	No FeLV or Rabies boosters. Fel. Panleuk. (2x) in 1996, 1998 and 2000 (2x)
FS III	ESH	12,5	mc	Between the shoulders	Fast: in a period of 5 months	Several times between the shoulders
FS IV	Persa	8	mc	Thorax	Not known	Not known

ESH: European short hair; FS: Fibrosarcoma; f: female; m: male; mc: male castrated; FeLV: Feline Leukemia Virus; Fel. Panleuk.: Feline Panleukopenia; 2x: twice

Annex 8.2: General details of the biopsy cases. Case number, breed, age (in years), sex, and tumor localization

Case No.	Breed	Age in years	Sex	Tumor localization
T502/08	ESH	7	Male	Back side
T675/08	ESH	9	Not known	Back
T918/08	Mixed breed	10	Male castrated	Neck
T2014/08	ESH	10	Female	Not specified
T3383/08	ESH	11	Male	Hind leg, thigh
T3462/08	ESH	6	Male	Back side
T4016/08	Mixed breed	10	Male castrated	Back side
T4047/08	Mixed breed	7	Male castrated	Between the shoulders
T5543/08	Mixed breed	12	Female	Back side
T5688/08	ESH	11	Female castrated	Between the shoulders
T6293/08	ESH	7	Male castrated	Hind leg
T6294/08	ESH	11	Female castrated	Not specified
T6403/08	ESH	Unknown	Female	Between the shoulders
T6781/08	ESH	12	Female castrated	Neck
T6817/08	ESH	14	Female castrated	Back
T7106/08	ESH	8	Male castrated	Back side
T7156/08	ESH	7	Female castrated	Back
T7259/08	ESH	10	Male	Neck
T7347/08	ESH	10	Male castrated	Thorax side
T7403/08	ESH	4	Female castrated	Abdomen side

ESH: European short hair.

Continuation of annex 8.2: General details of the biopsy cases. Case number, breed, age (in years), sex, and tumor localization

Case No.	Breed	Age in years	Sex	Tumor localization
T7405/08	ESH	13	Female castrated	Hind leg, thigh
T7908/08	ESH	5	Male castrated	Back side
T7939/08	Mixed breed	10	Male castrated	Head
T8642/08	ESH	12	Male castrated	Thorax side
T8650/08	ESH	8	Female castrated	Hind leg
T8692/08	ESH	8	Male castrated	Hind leg
T6035/04	ESH	6	Female	Hind leg, thigh
T8904/04	ESH	6	Female	Hind leg, thigh
T6125/05	ESH	7	Female	Hind leg, thigh
T8535/06	ESH	8	Female	Hind leg, thigh

ESH: European short hair.

Annex 8.3: Chromosomal analysis of the feline cell lines. The analysis of the chromosome number of the tumor cell lines was performed in another doctoral thesis at the Institut für Veterinär-Pathologie Justus-Liebig Universität Giessen (Löhberg-Grüne, 2009).

Cell Line	Chr./cell (Minimum)	Chr./cell (Median)	Chr./cell (Maximum)
FS I	27	47	64
FS II	27	47	113
FS III	23	66	144
FS IV	36	46	60
Control	34	38	38

Chr./cell: Chromosomes per cell.

Annex 8.4: Chemicals, Enzymes, Primers, Antibodies and Kits

Biozym, Oldendorf (Agent for Gentra Inc., Mineapolis MN, USA)

Puregene® Kit, Kat.-Nr.:202005

SeaKem® LE Agarose, Kat.-Nr.: 840.00

Carl Roth GmbH & Co, Karlsruhe

Ampicillin Natriumsalz, Kat.-Nr.: K029.1

DEPC ≥ 97 %. Kat.-Nr.: K028.1

Ethanol, Rotipuran® p.a., Kat.-Nr.: 9065.4

Formamid ≥ 99.5 %, Kat.Nr.: 6749.1

IPTG, Kat.-Nr.: 2316.2

LB-AGAR (Luria Miller), Kat.-Nr.: X969.2

LB-Medium (Luria Miller), Kat.-Nr.: X968.2

Proteinase K, Kat.Nr.: 7528.2

Roti®-Nylon plus, Kat.-Nr.: HP34.1

Tris-Puffer Ultra Pure, Kat.-Nr.: 5429.2

X-Gal, Kat-Nr.: 2315.3

Xylenzyanol, Kat.Nr.: C.I.2135

Dako Corporation, Carpintera

Fast Red substrate system, Kat.Nr.: K699

Diovana GmbH, Hamburg

Cy3-conjugated AffiniPure Goat Anti-Mouse IgG (H+L), 1.5 mg/ml,

Kat.Nr.: 115-165-003

Fluka Chemie GmbH, Buchs

Acetic anhydride ≥ 99.5 %, Kat.Nr.: 45830

Evans Blue, Kat.-Nr.: 4616

Paraformaldehyde 95 %, Kat.-Nr.: 76240

Invitrogen, Karlsruhe

Sybr Green (Sybr® safe DNA gel stain), Kat.-Nr.: S33102

Nagel Nunc International, Naperville

Lab-Tek II Chamber slide, Kat.-Nr.: 154526

Natutec, Frankfurt

BioTherm™ DNS-Polymerase, Kat.-Nr.: GC-002-002

Novagen-Merck Biosciences GmbH, Darmstadt

AccepTor™ Vector Kit, Kat.-Nr.: 01-1030

Macherey & Nagel, Düren

NucleoSpin® Extract II, Kat.-Nr.: 740590.250

NucleoSpin® Plasmid, Kat.-Nr.: 740588.250

MBI Fermentas, St. Leon-Rot

*Eco*RI, Kat.-Nr.: ER0271

Eco57MI, Kat.-Nr.: ER1671

6x Orange DNA loading dye solution, Kat.-Nr.: R0631

pUC19 DNA/MspI (HpaII), ready-to-use, Kat.-Nr.: SM0222

Merck, Darmstadt

HCl, 1M / l, Kat.-Nr.: 109970

Triton X-100, Kat.-Nr.: 108603

Millipore Corporate, Massachusetts

Microcon® centrifugal filter device YM-100, Kat.-Nr.: 42412

PAA Laboatories GmbH, Pasching, Austria

DMEM (Dulbecco's Modified Eagle's Medium), high glucose (4.5 g/l) with L-glutamine, Kat.-Nr.: E15-810

Roche, Mannheim

Anti-Digoxigenin-AP, Fab fragments 150 U (200 µl), Kat.-Nr.: 11093274910

Anti-Digoxigenin. Monoclonal antibody to digoxigenin from mouse-mouse-hybrid cell (clone 1.71.256), Kat.-Nr.: 11333062910

DIG RNA Labeling Kit (SP6/T7), Kat.-Nr.: 11175025910

Serva GmbH, Heidelberg

Fluoromount for microscopy, Kat.-Nr.: N21644

Sigma-Aldrich Chemie GmbH, Munich

Deoxyribonucleic acid sodium salt type III from Salmon testes, Kat.Nr.: D-1626

Hybridization Solution for In Situ Hybridization, Kat.-Nr.: H7782

Sheep Serum sterile-filtrated, Kat.-Nr.: S2263-100 ML

Annex 8.5: Solutions and Buffers

8.5.1 0.25 % Acetanhydride in 0.1 M Triethanolamine-DEPC-H_2O pH 7.5

894 mg Triethanolamine dissolved in 50 ml DEPC-H_2O

Adjust pH with 1 M HCl to 7.5

Add 150 µl Acetanhydride

Adjust volume to 60 ml with DEPC-H_2O

8.5.2 Blocking solution

1.2 ml Neutral sheep serum

1.8 ml 10 % Triton X-100

Adjust volume to 60 ml with „buffer 1"

8.5.3 Buffer 1, pH 7.5

12.11 g Tris (MW 121.14)

8.77 g NaCl (MW 58.44)

500 ml bi-distilled H_2O

Adjust pH with 1 M HCl to 7.5

Adjust volume to 1 l with bi-distilled H_2O

Autoclave at 121 ºC, 20 minutes

8.5.4 DEPC-H_2O Rnase free water

1 ml of Diethylpyrocarbonat (DEPC)

1 l of bi-distilled H_2O

Mix overnight

Autoclave at 110 ºC, 15 minutes

8.5.5 0.2 % Glycine-PBS

1 g Glycine (MW 75.07)

450 ml PBS pH 7.4

Adjust pH with 1M HCl to 7.4

Adjust volume to 500 ml with PBS pH 7.4

Autoclave at 121 ºC, 20 minutes

8.5.6 LB liquid culture medium

25g of medium ready to use, LB-Medium (Luria Miller) (Carl Roth, Karlsruhe)

1 l bi-distilled H_2O

Autoclave at 110 ºC, 15 minutes

Cool down to 55 °C add 100 µg/ml Ampicillin (Carl Roth, Karlsruhe)

Store at 4 °C until used

8.5.7 LB AGAR

40 g of medium ready to use LB-AGAR (Luria Miller) (Carl Roth, Karlsruhe)

1 l bi-distilled H_2O

Autoclave at 110 ºC, 15 minutes

Cool down to 55 °C add 100 µg/ml Ampicillin (Carl Roth, Karlsruhe)

Distribute on sterile petri dishes, let it solidify

Store at 4 °C until used

8.5.8 10 x TBE

108.8 g Tris (MW 121.14)

55 g Boric acid (MW 61.83)

8.3 g EDTA-Na_2 2 H_2O (MW 372.24)

1 l bi-distilled H_2O

Autoclave at 121 ºC, 20 minutes

8.5.9 4 % Paraformaldehyde, pH 7.4

40.0 g Paraformaldehyde

800 ml PBS pH 7.4

Dissolve at 60 °C on a magnetic stirrer

Adjust pH with 1 M NaOH to 7.4

Adjust volume to 1 l with PBS pH 7.4

8.5.10 10 x PBS, pH 7.4

80 g NaCl (MW 58.44)

2 g KCl (MW 74.56)

14.4 g Na_2-HPO_4 2 H_2O (MW 177.99)

2.4 g KH_2PO_4 (MW 136.09)

700 ml bi-distilled H_2O

Adjust pH with 1 M NaOH to 7.4

Adjust volume to 1 l with distilled H_2O

Autoclave at 121 ºC, 20 minutes

8.5.11 PBS, pH 7.4

100 ml of 10 x PBS

800 ml bi-distilled H_2O

Adjust pH with 1 M NaOH to 7.4

Adjust volume to 1 l with bi-distilled H_2O

Autoclave at 121 ºC, 20 minutes

8.5.12 PBS + 5 mM $MgCl_2$

10 ml 10 x PBS

5 µl 1 M $MgCl_2$

Adjust volume up to 100 ml with bi-distilled H_2O

8.5.13 Pre-hybridization solution

450 ml 20 x SSC

675 ml 100 % Formamid

150 ml 50 x Denhardts (5 g Ficoll, 5 g polyvinylpyrrolidone, 5 g bovine serum albumin, fill up to 500 ml with bi-distilled H_2O, autoclave at 121 ºC, 20 minutes.)

210 ml bi-distilled H_2O

Mix and aliquot

Store at - 20 °C

8.5.14 Proteinase K reaction solution

1 ml 1 M Tris pH 8.0

1 ml 0.1 $CaCl_2$

Adjust volume to 60 ml with DEPC-H_2O, mix

8.5.15 20 x SSC pH 7.0

175.3 g NaCl (MW 58.44)

88.2 g Tri-Na-Citrate x 2 H_2O (MW 294.1)

800 ml bi-distilled H_2O

Adjust pH to 7.0 with 1 M HCl

Adjust volume to 1 l with bi-distilled H_2O

Autoclave at 121 ºC, 20 minutes

8.5.16 2 x SSC + 5 mM EDTA-Na_2

50 ml 20 x SSC pH 7.0

5 ml 0.5 M EDTA-Na_2

Adjust volume to 500 ml with bi-distilled H_2O

Autoclave at 121 ºC, 20 minutes

8.5.17 40 x TAE

193.6 g Tris (MW 121.14)

108.8 g Boric acid (MW 61.83)

8.3 g EDTA-Na_2 2 H_2O (MW 372.24)

Adjust volume to 1 l with bi-distilled H_2O

Autoclave at 121 ºC, 20 minutes

8.5.18 TBS, pH 7.6

60.57 g Tris (MW 121.14)

610 ml bi-distilled H_2O

390 ml 1 M HCl

Adjust pH with 1 M HCl

8.5.19 1 M Tris pH 8.0

12.11 g Tris (MW 121.14)

50 ml bi-distilled H_2O

Adjust pH with 1 M HCl to 8.0

Adjust volume with bi-distilled H_2O to 100 ml

Autoclave at 121 ºC, 20 minutes

Curriculum vitae

26th February 1977	Born in San José, Costa Rica
1990 - 1995	José Joaquin Vargas Calvo High School
1996 - March 2004	Studies and degree in Veterinary Medicine, College of Veterinary Medicine, Universidad Nacional de Costa Rica
2002	Externship at the dermatology department and the oncology department, North Carolina State University, College of Veterinary Medicine, USA
2004 March - December	Small animal practice
2005 - October 2006	Pathology Department at the College of Veterinary Medicine, Universidad Nacional de Costa Rica
2006 January - March	Externship at the Institut für Veterinär-Pathologie, Tierärztliche Hochschule Hannover, Germany
October 2006 - 2009	PhD student and resident in veterinary pathology at the Institut für Veterinär Pathologie der Justus-Liebig-Universität Giessen, Germany

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