

FGF4 and INT2 Oncogenes Are Amplified and Expressed in Kaposi's Sarcoma

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Kaposi's sarcoma (KS) is a vascular tumor, the pathogenesis of which has been suggested to include human herpesvirus 8 (HHV-8) as well as various cytokines and growth factors. Very little is known about cytogenetic and molecular genetic changes in KS. We studied DNA copy number changes in KS and found a recurrent gain at 11q13. We then analyzed the amplification and expression status of two known oncogenes, *FGF4* and *INT2*, residing at 11q13. Comparative genomic hybridization, interphase fluorescence *in situ* hybridization with yeast artificial chromosome probes containing *FGF4* and *INT2*, and immunoperoxidase immunostaining with anti-*FGF4* and -*INT2* antibodies were used on 12 KS samples. All samples tested were shown by polymerase chain reaction to be HHV-8 positive. A recurrent gain at 11q13 was shown by comparative genomic hybridization in 4 of 10 cases studied. Of six cases studied by interphase fluorescence *in situ* hybridization, four showed a 3- to 4-fold amplification with the probes containing *FGF4* and *INT2*. Expression of *FGF4* and *INT2* was found in nine and three cases, respectively, of nine studied. Amplification and expression of these genes is particularly interesting in the context of oncovirus involvement, because *INT2* is a homolog of mouse *int2*, which causes mammary carcinoma in mice when activated by integration of retrovirus mouse mammary tumor virus. This raises the question of whether HHV-8 represents an integrating oncovirus that causes amplification and activation of genomic oncogenes in humans.

KEY WORDS: Comparative genomic hybridization, DNA amplification, *FGF4*, Human herpesvirus-8, *INT2*, Kaposi's sarcoma.

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Kaposi's sarcoma (KS) is a vascular tumor characterized by proliferation of endothelial cells and spindle cells accompanied by lymphoplasmacytic infiltration (1). In patients who are seropositive for the human immunodeficiency virus (HIV), KS is the most common malignant lesion but also occurs in patients who experience iatrogenic immunosuppression and endemically in some African and Mediterranean populations (2). Because of its often indolent course and common tendency to spontaneously regress, KS has been suggested to be a hyperplastic rather than a neoplastic process (3, 4). During the past few years, a γ -herpesvirus, human herpesvirus 8 (HHV-8), or Kaposi's sarcoma-associated herpesvirus, has been consistently demonstrated in HIV-associated and unassociated KS and is believed to be pathogenetically important in KS (5-8). Multiple factors have been suggested to contribute to the angioproliferation and transformation of KS cells. These include angiogenic and growth factors such as VEGF/VPF, FGFs, and so forth (9, 10). Very little is known about cytogenetic and molecular genetic changes in KS. To trace DNA copy number changes in KS, we applied comparative genomic hybridization (CGH) and found a recurrent gain at 11q13. Multiple genes reside in this amplicon, including two oncogenes of possibly special interest in KS: *FGF4* (*HSTF* or *K-FGF*), which has originally been identified by transfection of KS DNA (11), and *INT2* (*FGF3*), whose mouse homolog is activated by provirus insertion in murine mammary carcinogenesis. Here we have studied the potential roles of *FGF4* and *INT2* in KS.

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MATERIALS AND METHODS

Samples

Twelve histologically verified KSs were studied. The gender and AIDS or HIV status of the patients are shown in Table 1. All tissues were fixed in formaldehyde and embedded in paraffin and were further processed as described next.

Polymerase chain reaction by HHV-8 sequence-specific primers

HHV-8-specific DNA was amplified by polymerase chain reaction (PCR) using the forward and reverse primers 5'-ggagggcagcgtactctcagtg and 5'-tcctcactccaatccaatgc, respectively (modified from Muralidhar *et al.* [12]), that are specific for the K12 open reading frame, also known as kaposin. The kaposin gene was recently identified as an HHV-8 transforming gene (12).

Six KS samples, 10 epithelioid sarcoma samples, 1 dermatofibrosarcoma protuberans sample, and water were used as templates in the PCR after the DNA had been extracted from paraffin sections as described by Miller *et al.* (13) and were diluted in water 1:10 and 1:100. PCR cycles were 94° C for 10 min to activate the enzyme, then 30 × 94° C for 15 seconds, 55° C for 30 seconds, and 72° C for 40 seconds, then 72° C for 5 min in a reaction mixture containing 200 μM each dNTP, 0.6 μM each primer, and 2.5 units Ampli Taq Gold polymerase in the buffer supplied by the manufacturer (Perkin Elmer Applied Biosystems, Foster City, CA).

The amplified products were separated in gel electrophoresis, transferred to nylon membrane, and hybridized to a digoxigenin-labeled probe. The probe was made by incorporation of DIG-11-dUTP (Boehringer-Mannheim, Mannheim, Germany)

into the synthesized DNA strands by PCR, using the same primers and conditions as above.

Comparative genomic hybridization

Representative areas of the purest available tumor tissue were chosen from each case for CGH. Paraffin sections from these areas were cut into tubes, and DNA was extracted as described by Miller *et al.* (13). Ten samples yielded DNA with satisfactory strand length. CGH was performed using fluorochromes conjugated to a mixture of dCTP and dUTP for standard nick translation (14). Briefly, the tumor DNA was labeled with fluorescein isothiocyanate -dCTP and -dUTP mixture (DuPont, Boston, MA), and reference genomic DNA was labeled with Texas red -dCTP and -dUTP mixture (DuPont) by nick translation to obtain DNA fragments ranging from 600 to 2000 base pairs. The hybridization mixture consisted of 800 ng labeled tumor DNA, 800 ng labeled reference DNA, and 20 μg COT-1 DNA in 10 μl hybridization buffer (50% formamide, 10% dextran sulfate, 2 × standard saline citrate [SSC]). The hybridization was performed after 5 min denaturation at 75° C of the hybridization mixture and 2 min denaturation of the metaphase spread slides in 70% formamide/2 × SSC at 68° C.

After 48 h of hybridization at 37° C, the slides were washed three times in 50% formamide/2 × SSC (pH 7), twice in 2 × SSC, and once in 0.1 × SSC at 45° C, followed by 2 × SSC, 0.1 M NaH₂PO₄-0.1 M Na₂HPO₄-0.1% NP40 (pH 8), and distilled water at room temperature for 10 min each. After air drying, the slides were counterstained with 4',6-diamidino-2-phenylindole-dihydrochloride (Sigma, St. Louis, MO) and then mounted with an antifading medium (Vectashield, Vector Laboratories, Burlingame, CA).

TABLE 1. Summary of the CGH, FISH, Immunohistochemical Staining, and HHV-8-Specific PCR Results of 12 KS Cases

Case	Sex/HIV	Copy Number Changes	Number of FISH Signals		Immunoreactivity of Tumor Cells		HHV-8 PCR
			YAC 55G7 (Range)	YAC 214D11 (Range)	FGF4	INT2	
1	F/-	+11q13	3 (2-5)	3 (2-5)	-	-	NA
2	M/HIV	+11q13	NA	NA	+	-	+
3	M/-	+11q13, +22	3 (2-5)	3 (2-6)	NA	NA	NA
4	M/HIV	+11q13, +16p	NA	NA	+	-	NA
5	F/-	-6q24-qter, +8q, -10, +16p	3 (2-5)	3 (2-5)	++	++	NA
6	M/AIDS	NR	4 (2-5)	4 (2-6)	+	+	NA
7	M/AIDS	NR	4 (2-6)	3 (2-6)	+	-	+
8	M/AIDS	None	2	2	++	+	+
9	M/-	None	NA	NA	++	-	+
10	M/-	None	NA	NA	NA	NA	+
11	M/-	None	NA	NA	NA	NA	NA
12	M/AIDS	None	2	NR	+	-	+

CGH, comparative genomic hybridization; FISH, fluorescence *in situ* hybridization; HHV-8, human herpesvirus 8; PCR, polymerase chain reaction; KS, Kaposi's sarcoma; HIV, human immunodeficiency virus; YAC, yeast artificial chromosome; F, female; M, male; NR, unsuccessful hybridization; NA, no sample available.

Digital image analysis

The hybridizations were analyzed using an Olympus fluorescence microscope and the ISIS digital image analysis system (MetaSystems GmbH, Altlussheim, Germany) based on an integrated high-sensitivity monochrome charge-coupled device camera and automated CGH analysis software. Three-color images—red for reference DNA, green for tumor DNA, and blue for counterstaining—were acquired from five to nine good quality metaphases for each sample. The green and red fluorescence intensities were calculated, and the red-to-green ratio profiles along the chromosome axis were displayed. Chromosomal regions were interpreted as overrepresented when the corresponding ratio exceeded 1.17 (gains) and as underrepresented (losses) with a ratio of less than 0.85. The results were confirmed using 99% confidence interval.

Interphase fluorescence in situ hybridization

Two overlapping yeast artificial chromosome (YAC) clones, 55G7 and 214D11, covering the *FGF4/INT2* region at 11q13, were used as probes (15). YAC 950, which hybridizes to 11p16, was used as a control probe. The probes, labeled with biotin-14-d-ATP using standard protocols, were visualized with fluorescein isothiocyanate. The probes gave specific hybridization signals in normal metaphase preparations at the expected locations.

Interphase fluorescence *in situ* hybridization (FISH) was performed in six cases (Table 1) on nuclei extracted from paraffin sections as described previously (16). Paraffin-embedded reactive lymphatic tissue was used as control target. When all probes were hybridized separately on these nuclei, they showed two signals in more than 73% of the cells.

Immunohistochemistry

Formalin-fixed and paraffin-embedded KS samples were immunohistochemically evaluated with a monoclonal antibody FGF4 (dilution 1:1000) (Sigma Chemical, St. Louis, MO) and polyclonal antibody FGF3 (INT2) (dilution 1:50) (Santa Cruz Biotechnology, Santa Cruz, CA). Avidin-biotin complex immunoperoxidase technique was performed by using Elite ABC kit (Vectain, Vector Laboratories). Normal skin surrounding the tumor was used as a control.

RESULTS

PCR for HHV-8

All six KS samples tested were HHV-8 K12 positive (Table 1), yielding an expected 222 bp fragment in gel electrophoresis, whereas all negative control samples were negative. Southern hybridization confirmed the results.

CGH

CGH was successful in 10 of 12 cases (Table 1). Four of them (Cases 1, 2, 3, and 4) showed gains in 11q13, which was the only change in two cases (1 and 2). The additional changes were gains of chromosome 22 (Case 3), 16p (Case 4), 8q, and 16p (Case 5), as well as losses of 6q24-qter and chromosome 10 (Case 5). The copy number changes are shown graphically in Fig. 1. Five cases showed no DNA copy number changes.

Interphase FISH

Interphase FISH studies were performed in seven cases (1, 3, 5, 6, 7, 8, and 12). Case 8 consistently gave two signals with both probes, and Case 12 gave two signals with 55G7 (FISH with 214D11 was not successful). Cases 1, 3, and 5 showed on average three signals with both probes; the number of signals between different cells varied from two to six. Case 6 gave on average four signals with both probes (range, 2 to 6). Case 7 gave on average four (range, 2 to 6) and three (range, 2 to 6) signals with 55G7 and 214D11, respectively (Fig. 2). The control probe 950 gave two signals in 78% of the cells.

Immunohistochemistry

The results are summarized in Table 1. Three cases showed significant FGF4-reactivity, which was observed in at least 30% of spindle cells and endothelial cells of the KS lesions. Five additional cases showed focal or patchy reactivity in up to 10% of tumor cells. Two cases were negative. Among the non-neoplastic components, some pericytes, smooth muscle cells of the vessel walls, arrector pili smooth muscle cells, basal cells of epidermis, and occasional inflammatory cells were positive.

INT2-immunoreactivity was observed in three cases that were also positive for FGF4 in a similar manner. One of the positive cases showed strong,

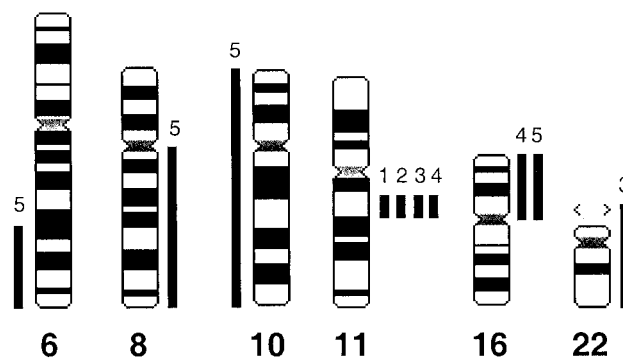


FIGURE 1. Summary of gains and losses of DNA sequence copy number in 10 Kaposi's sarcomas by comparative genomic hybridization. Losses are shown to the left and gains to the right of the chromosomes. The numbers above the bars refer to the case numbers shown in Table 1.

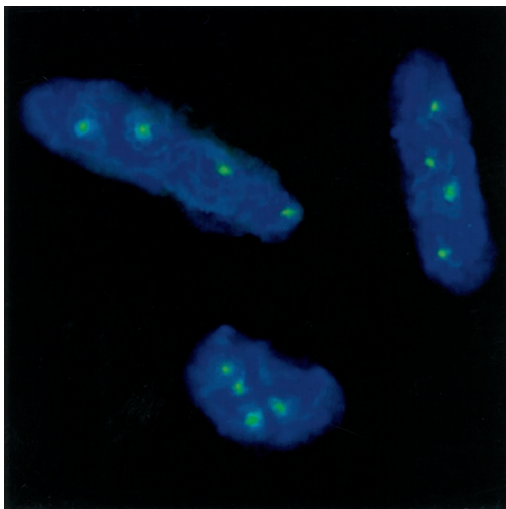


FIGURE 2. Interphase fluorescence *in situ* hybridization of Case 7 with probe 55G7. Green fluorescein isothiocyanate signals in the cells indicate a 2-fold amplification in different cells.

widespread reactivity (Fig. 3), and in two cases, up to 10% of the tumor cells were focally positive. Among the non-neoplastic components, the only ones to show positivity were occasional basal cells in the epidermis and hair shaft epithelium in one case.

DISCUSSION

In the present study, we demonstrated a DNA sequence copy number gain at 11q13, showed amplification of two probes carrying the *FGF4* and *INT2* genes, and indicated expression of these genes in KS. The gain at 11q13 is frequently observed in human malignancies, such as breast cancer, head and neck and oral squamous cell carcinomas, small cell lung cancer, and carcinomas of the urinary bladder (17). In addition to the oncogenes *FGF4* and *INT2*, *CCND1* and *EMS1* have been assigned to this amplicon (18–20). The *CCND1* and

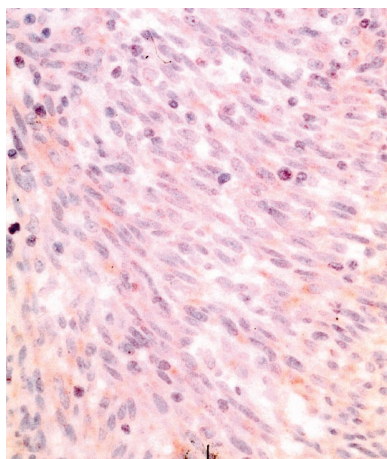


FIGURE 3. Immunohistochemical staining of Case 5 with anti-*FGF4* antibody shows strong, widespread immunoreactivity.

EMS1 genes may also be amplified at the 11q13 gain, but the status of amplification and expression was studied only for *FGF4* and *INT2* because of their theoretical significance.

The absence of detectable DNA copy number changes in five KS cases may have been caused by the inevitable presence of non-neoplastic cells in the tumors. The non-neoplastic leukocytes, fibroblasts, and so forth may dilute the DNA copy number changes in tumor cells beyond the resolution of CGH. In addition, to be detected by CGH, the total amount of amplified DNA has to be at least 2Mb; thus, a 3- to 4-fold amplification of a single gene inevitably would remain below the detection limit of CGH (21). Indeed, expression of at least *FGF4* was detected in all but one case studied by immunohistochemistry. Expression was present in one case with other detectable CGH changes but gain at 11q13. This is probably an indication of the limited resolution capability of CGH; amplification was seen with both probes in this case. However, one case with a DNA copy number gain at 11q13, also shown to be amplified with both probes, failed to show immunoreactivity with either antibody. This may be caused by the lack of antigen preservation, by nonspecific staining reaction, or by other mechanisms not fully understood, leading to negative immunohistochemical staining result. In the mouse, *fgf4* and *int2* are known to be expressed during embryogenesis (22, 23). Assuming the expression pattern to be similar in humans, no expression would be expected in normal adult tissues. In some samples, however, some weak positivity was seen also among occasional non-neoplastic cells. This most likely nonspecific staining is probably due to the primary fixation process.

FGF4 and *INT2* belong to the fibroblast growth factor family, members of which have been suggested to be involved in the pathogenesis of KS (10, 24). Coamplification of *FGF4* and *INT2* has been reported in different human malignancies including melanoma, breast cancer, and oral squamous cell carcinoma, but the expression pattern varies (25–27). *FGF4* was originally identified by transfection of stomach, colon cancer, and KS DNAs into NIH3T3 cells (11, 28). It is a potent mitogen for different mesodermal cell types, including fibroblasts and endothelial cells. *INT2* is a homolog of murine mammary tumor virus integration site oncogene *int2*, which is activated in some mouse mammary carcinomas by integration of murine mammary tumor virus proviruses. Overexpression of either *FGF4* or *INT2* has been demonstrated to induce tumorigenesis in EF43 mouse cells (29, 30). Expression of *INT2* has been reported in KS lesions, but the activation mechanism is not known (31). Our data show that not only *INT2* but also *FGF4*, to even greater excess, is expressed in KS cells. The

mechanisms of amplicon formation are not well understood, but our FISH results suggest gene amplification to be the probable cause of the expression, although mechanisms such as activating mutations cannot be excluded. The identification of oncogenes in KS with homology to mouse genes known to be activated by a tumor virus is intriguing, considering the recognition of HHV-8 as the causative agent of KS. Although no evidence of HHV-8 integration to the genome has been shown, this cannot be ruled out as a possible mechanism of amplification and oncogene activation in KS and needs further investigation.

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