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Restriction Fragment Length Polymorphism of the c-*fms* Gene in the Human Oral Squamous Cell Carcinomas

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ABSTRACT

A restriction fragment length polymorphism (RFLP) for the c-*fms* gene was identified in the human oral squamous cell carcinoma cell lines, Ca9–22, HSC–2 and –3. The RFLP was detected after *Eco*R I, *Bam*H I and *Hin*d III endonuclease digestion, indicating the presence of two alleles, *a* and *b*. The allele *a* deleted 426bp length of allele *b*. We determined the sequence of this deletion, that localized in intron 11 with an *Eco*R I site. The phenotype of Ca9–22 was *aa*, and the others were *bb*. Both phenotypes were equally expressed and the transcripts were phosphorylated in these cell lines. The distribution in the analyzed population (66 patients and normal individuals) was 3.1% homozygotic *aa*, 13.5% heterozygotic *ab* and 83.4% homozygotic *bb*.

Key words: *c-fms oncogene, Restriction fragment length polymorphism (RFLP), Oral carcinomas*

Oncogenes have been rather well conserved during evolution. Hence, polymorphism is probably not frequent among them. Detection of a polymorphism for a specific oncogene might, therefore, have some consequences when the panel of DNA samples studied is able to answer to many other genetic markers. In this context, we planned a restriction fragment length polymorphism(RFLP) analysis of the c-*fms* gene in the human oral squamous cell carcinomas.

The c-*fms* proto-oncogene encodes the cell surface receptor for the macrophage colony stimulating factor, CSF–1[18]. This proto-oncogene is homologous to the viral oncogene v-*fms* of the McDonough strain of the feline sarcoma virus(SM-FeSV[3]). Conceptual translation of the nucleotide sequences of the feline v-*fms* oncogene[4] and the human c-*fms* proto-oncogene cDNA[2] predicted that their encoded products are: integral transmembrane glycoproteins composed of an extracellular ligand-binding domain, a single hydrophobic membrane-spanning segment, and an intracellular tyrosine-specific protein kinase domain.

The c-*fms* proto-oncogene maps near the CSF–1 locus on human chromosome 5 at band 5q33.3[14] relatively close to other growth factors and receptor genes that play important roles in hematopoiesis. These include the GM-CSF, IL–3, IL–4, IL–5 and PDGF-R_B genes. The c-*fms* gene is about 60kb long, containing 22 exons and heterogeneous introns[5,17].

We recently observed a RFLP for c-*fms* gene in human oral squamous cell carcinoma cell line Ca9–22[7] by means of Southern hybridization probed with v-*fms* DNA. Walker et al[21] reported that the expression of a *fms*-related oncogene was altered in carcinogen-induced neoplastic epithelial cells. It was also reported that one human oral squamous cell carcinoma cell line produced colony-stimulating factor[10]. Then we suspected the relation between the c-*fms* gene and oral carcinomas. Here we describe the RFLP for the c-*fms* gene and the expression of a glycoprotein with its tyrosine kinase activity in human oral squamous cell carcinomas.

MATERIALS AND METHODS

Cell lines: The human oral squamous cell carcinoma cell lines (Ca9–22, HSC–2 and –3 were conferred by JCRB) were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum[7,8].

DNA extraction and Southern blotting: High-molecular-weight cellular DNA was extracted using proteinase K and phenol/chloroform[16]. DNA, 10μg, was digested with restriction enzymes according to the manufacture's instructions. The resulting fragments were fractionated by electrophoresis in 0.7% agarose gels and transferred to the nitrocellulose filters[16]. The *Pst* I-*Bgl* II fragment (0.97kb) of the v-*fms* gene (TAKARA) encoding the 3'portion of SM-FeSV proviral DNA and the *Eco*R I-*Hin*d III fragment(0.9kb) of the human c-*fms* gene (from Oncogene Science) isolated from the cosmid clone Cos 10–2[6] were used as probes and labeled by the random primer method with [α–^{32}P] dCTP (>3000Ci/mmol, Amersham) to

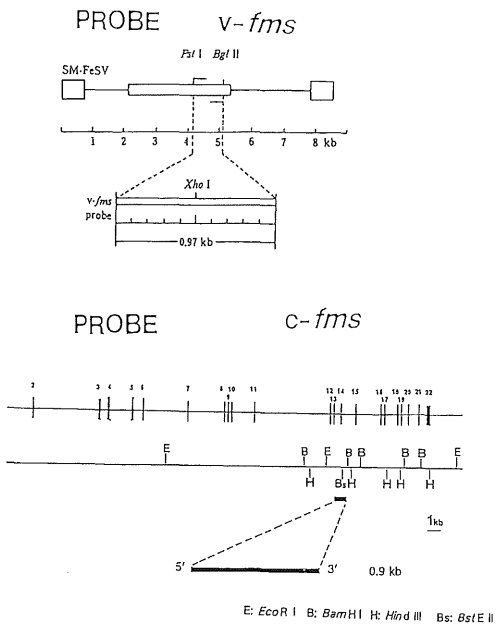

Fig. 1. DNA probes: the *Pst* I-*Bgl* II fragment (0.97kb) of v-*fms* gene encoding the 3' portion of SM-FeSV proviral DNA and the *Eco*R I-*Hin*d III fragment (0.9kb) of the human c-*fms* gene isolated from cosmid clone Cos 10–2

a specific activity $>10^8$cpm/μg (Fig. 1).

Prehybridization proceeded for 2–12hr at 42°C in 50% deionized formamide/6xSSC (1x=0.15M NaCl, 0.015M sodium citrate) /5xDenhardt's solution (1x= 0.02% bovine serum albumin/ 0.02% polyvinylpyrrolidone/ 0.02% Ficoll) /0.5% sodium dodecyl sulfate (SDS) /10mM EDTA (pH 8.0)/ 0.1mg/ml sonicated salmon sperm DNA (DNA prehybridization buffer). Hybridization proceeded for 12–24hr at 42°C in the DNA prehybrydization buffer containing 10% dextran sulfate and the radioactive probe at 25ng/ml. After hybridization, the filters were washed with 2xSSC at room temperature, and 0.5xSSC/ 0.1%SDS at 42°C, followed by autoradiography on Fuji AR x-ray films with intensifying screens for 4–48hr at –70°C.

RNA extraction and Northern blotting: Total RNA was extracted with guanidine-HCl[16], then fractionated by electrophoresis in 1% agarose gels and transfered to nitrocellulose filters. Prehybridization proceeded for 2–12hr at 42°C in 50% formamide/ 0.65M NaCl/ 0.1M sodium Pipes (pH 6.8)/ 5xDenhardt's solution/ 0.1% SDS/ 5mM EDTA/ 0.1mg/ml denatured salmon sperm DNA (RNA prehybridization buffer). Hybridization followed in the RNA prehybridization buffer containing 10% dextran sulfate and the radioactive probe at 25ng/ml. The filters were washed with 2xSSC/ 0.1% SDS/ 0.2% sodium pyrophosphate at 42°C, and were autoradiographed as described above.

Endogenous immune-complex kinase assay: Subconfluent cultures of the cell lines were lysed with 2ml RIPA buffer (50mM Tris-HCl(pH 7.4) containing 150mM NaCl, 20mM EDTA, 1% TritonX–100, and 1% sodium deoxycholate) containing 2% Aprotinin and 1mM phenylmethylsulfonyl fluoride as protease inhibitors. Nuclei and debris were removed by centrifugation. The lysates were divided into 1ml portions, SDS was added to a final concentration of 0.1%. Immune complexes were prepared by incubating the cell lysate with the rabbit c-*fms* polyclonal antiserum (CAMBRIDGE RESEARCH BIOCHEMICALS) for 30min at 22°C and overnight at 4°C. *Staphylococcus aureus* protein-A-sepharose was added, and immune complexes were collected by centrifugation, washed 5 times with RIPA buffer containing 0.1% SDS and 2mM EDTA, then washed twice with 50mM Tris-HCl (pH 7.4). Kinase reactions were initiated by adding 50mM Hepes buffer (pH 7.4) containing $MnCl_2$, 1% TritonX–100, and 20μCi/ml adenosine [γ–^{32}P] 5' triphosphate ([γ–^{32}P] ATP; 7000mCi/mmol). The precipitates were suspended and incubated for 10min at 30°C. Reactions were terminated by adding electrophoresis sample buffer followed by heating at 100°C for 2min. The ^{32}P-labelled products were separated by electrophoresis in 7.5% SDS-polyacrylamide slab gels and were detected by autoradiography[11,12].

Cloning the c-fms RFLP fragment and DNA sequencing: We enzymatically amplified specific segments of genomic DNA and directly cloned them into M13 vectors for sequence analysis, using a modified polymerase chain reaction (PCR)[15]. Oligonucleotide primers for PCR amplification were synthesized using a DNA synthesizer. The sequences of oligonucleotide primers used to detect the c-*fms* RFLP were 20mer oligonucleotides containing 2 base changes to generate *Bam*H I and *Hin*d III sites. The primers were 5' GGATCCGCTACTATTATTCA 3' and 5' TGTAAGCTTCCCTTCTCCAA 3'. DNA was amplified by mixing 1μg of genomic DNA in the polymerase buffer (50mM KCl, 10mM Tris-HCl (pH 8.3), 1.5mM $MgCl_2$, 0.01% gelatin) with 500pmol of each primer, 200μM dNTPs and 2.5units Taq DNA polymerase (Perkin Elmer Cetus) in a total volume of 100μl. The mixture was incubated during 40 cycles for 1–5min at 94°C for denaturation, 2min at 55°C for annealing and 3–10min at 72°C for polymerization. After amplification, PCR products were digested with *Hin*d III and *Bam*H I or blunt-ended with T4 DNA polymerase and phosphorylated with T4 DNA kinase. The modified fragments were ligated to *Hin*d III/*Bam*H I or *Sma* I cut M13mp18 cloning vectors under standard conditions[15]. The ligation mixture was transformed into competent *Escherichia coli* JM109 and plated on IPTG/X-gal agar plates. After overnight incubation at 37°C, single-

stranded DNA was prepared from colourless plaques and sequenced by dideoxy chain termination (TOYOBO).

A c-fms RFLP survey of oral cancer patients: DNA samples, derived from tumor specimens and/or peripheral blood were extracted, digested with *Bam*H I, and hybridized with the c-*fms* probe described above.

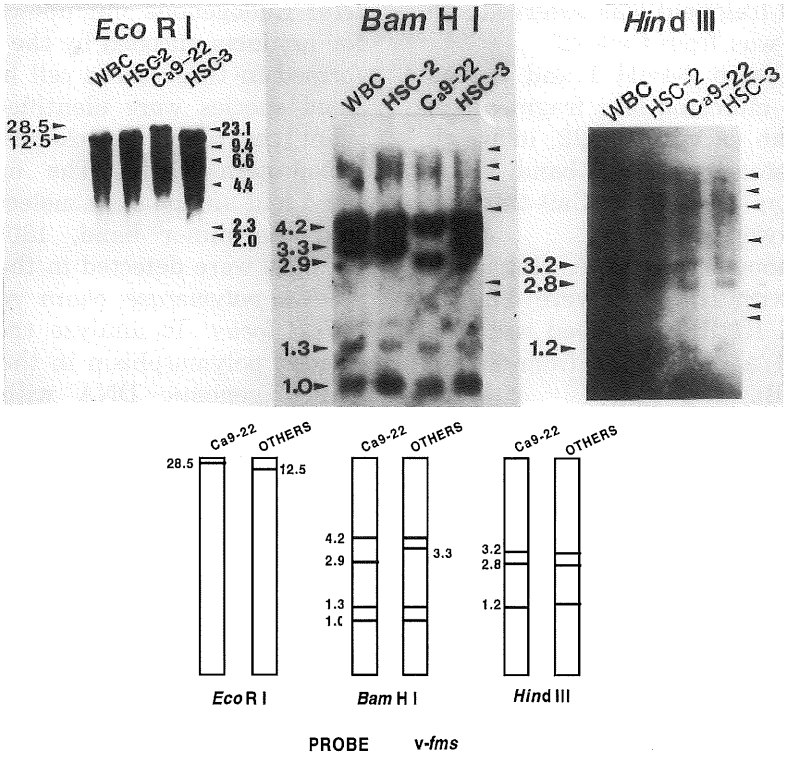

Fig. 2A. Autoradiograms and a schematic representation of *Eco*R I, *Bam*H I and *Hin*d III digests of DNAs hybridized with v-*fms* DNA probe

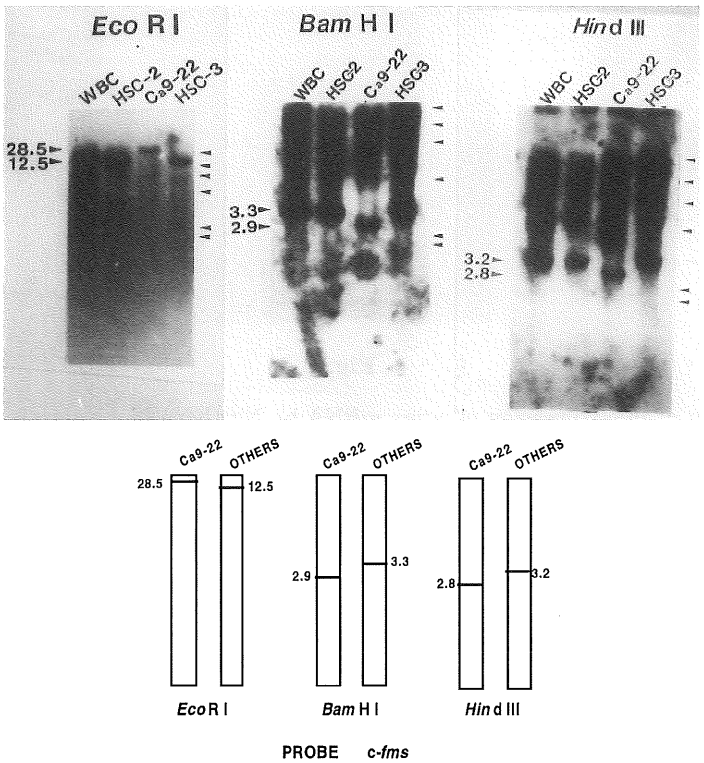

Fig. 2B. Autoradiograms and a schematic representation of *Eco*R I, *Bam*H I and *Hin*d III digests of DNAs hybridized with c-*fms* DNA probe

RESULTS

RFLP detection: The *Eco*R I restriction profiles, obtained by Southern hybridization with the v-*fms* and c-*fms* probes, indicated one DNA fragment. Among the DNA isolated from control normal human peripheral blood (WBC), HSC–2 and –3, the 12.5kb *Eco*R I fragment was observed and the 28.5kb fragment was from Ca9–22.

The DNA digested with *Bam*H I and probed with the v-*fms* DNA, indicated four fragments of 4.2, 3.3, 1.3 and 1.0kb except Ca9–22, in which 3.3kb band was replaced by a 2.9kb band. When probed with the c-*fms* DNA, 2.9 (Ca9–22) and 3.3kb (others) bands were revealed.

The v-*fms* probe homologous DNA restriction fragments digested with *Hin*d III were 3.2, 2.8 and 1.2kb. The *Hin*d III RFLP, probed with the c-*fms* DNA, revealed a band shift from 3.2 to 2.8kb (Fig. 2 A and B).

Expression of c-fms mRNA: Northern blotting showed that the 0.97kb *Pst* I-*Bgl* II v-*fms* probe DNA hybridized about 4.0kb RNA in these three cell lines. The β-actin mRNA, used as an internal control, hybridized about 2.5kb RNA. No cell lines overexpressed or had altered lengths of c-*fms* mRNA (Fig. 3).

Tyrosine kinase activity of the fms-encoded protein: Endogenous immune-complex kinase assay for products encoded by the c-*fms* gene, was performed in these three cell lines. Two phosphorylated species were identified in a kinase assay using the rabbit polyclonal antibody to the *fms* oncoprotein (CRB). The major phosphorylated band had an apparent molecular mass of 150kDa, and the minor band, 130kDa. No significant changes were detected in these cell lines (Fig. 4).

DNA polymerase chain reaction of the c-fms RFLP locus: To analyze the molecular basis of genetic polymorphism in the c-*fms* gene, we amplified genomic DNA with 20 oligonucleotide primers containing 2 base changes to generate *Bam*H I and *Hin*d III sites (Fig. 5). Electrophoresis and ethidium bromide staining the agarose gel, showed amplified fragments of control human peripheral blood WBC, HSC–2 and –3 that were about 580 and 490bp long, and those of Ca9–22 were about 490 and 160bp long (Fig. 6).

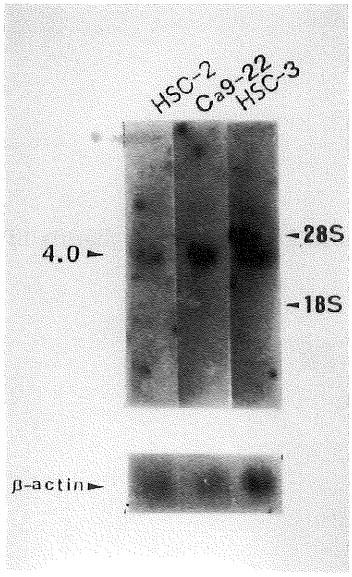

Fig. 3

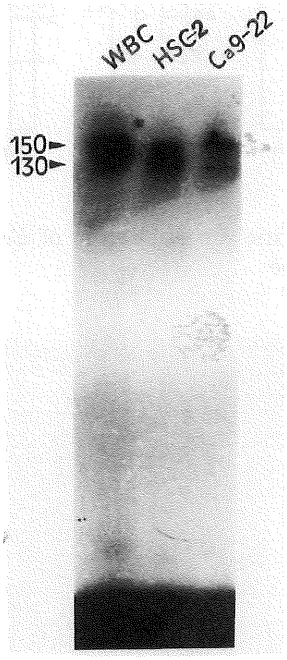

Fig. 4

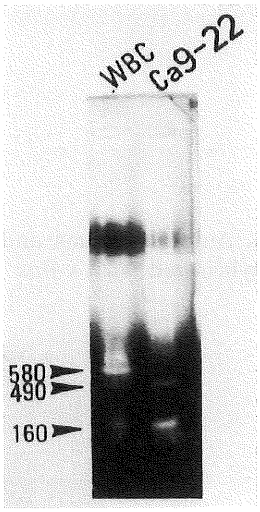

Fig. 6

Fig. 3. Autoradiogram of the expression of c-*fms* mRNA by Northern blot analysis with the v-*fms* probe DNA and the β-actin probe DNA as internal control
Fig. 4. Endogeneous immune-complex kinase assay for products encoded by the c-*fms* gene
Fig. 6. Ethidium bromide staining of the amplified fragments by electrophoresis in a 1% agarose gel

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PRIMER(F)    5'-GGATCCGCTACTATTATTCA-3'
PRIMER(R)    5'-TGTAAGCTTCCCTTCTCCAA-3'
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Fig. 5. Sequence of oligonucleotide primers containing 2 base changes to generate a *Bam*H I site and a *Hin*d III site (underlined)

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        10         20         30         40
GGATCCGCTACTATTATTCAGCCTTAAA AAGGAAGGGAAT
        50         60         70         80
TCTGACCTGTGCTGCAGCATGAATGAACCTTGAAGACATT
        90        100        110        120
ATGCTGGGTGAAATAAGGCAATCTCAATAGACACATGCTG
       130        140        150        160
TGTGAGTCCACTGAGGTGCAGTGCCTAGAGCAGTGCAATT
       170        180        190        200
CACAGAGACAGCAGAATCATGGTTCGCCAGGGGCTGGAGG
       210        220        230        240
AGGGAAAGGGGAGTTGCTTTTTAACAGGAACAGAATCTCA
       250        260        270        280
GTTTTGCAAGATGAAAAGAGCTCTGGAAACTGGTTGCACA
       290        300        310        320
AGGTAGAATGTAATTTACTTAATACTACTGAACCATACAC
       330        340        350        360
TTAAAAATGGTTGAAATGGTAAATTTCATGTATGTTTTAT
       370        380        390        400
CACAATTAAAATATATATATATATTTGGATGGGAGGTTGG
       410        420        430        440
GTGGGTGGATGGATGGGTAGATGGATGGACAGATGAACGG
       450        460        470        480
ATGGATAAGATCTC AAGTTCCACCCTCCCTCCTGGCTCAG
       490        500        510        520
GAATTACCAGATTATCAGAGATATCAGGGCCCTCAGAGGT
       530        540        550        560
TGTCTTGTCCAAGGTCTTCAATACACAAATAGTGAAACAG
       570        580        590        600
GCTTGGAGAAGGGAAGCTTACA
```

Fig. 7. Sequence of the deletion of 428bp segment localized in intron 11

Molecular cloning and sequencing the c-fms RFLP fragment: Following polymerase chain reaction, amplified 580 and 160bp fragments were ligated into the M13mp18 cloning vector and sequenced by dideoxy chain termination. Sequencing showed that the genetic change of Ca9–22 resulted in a deletion of 426bp in length located close to exon 12. The start and end of the deleted sequence was AAG (Fig. 7).

A c-fms RFLP survey of oral cancer patients: Southern blot hybridization with the c-*fms* probe, revealed a polymorphism for *Bam*H I in samples from oral cancer patients. This RFLP could be explained by the presence of two alleles, *a* and *b*. The former was the result of 426bp deletion of allele *b*. The distribution in the analysed population was 13.5% heterozygotic *ab*, 3.1% homozygotic *aa*, and 83.4% homozygotic *bb* (Table 1).

Table 1. Frequency of the RFLP of the c-*fms* gene

	Genotype (%)		
	aa	*ab*	*bb*
Tumors			
SCC*	0 (0)	1 (4.2)	23 (95.8)
Others	1 (4.6)	5 (22.7)	16 (72.7)
WBC	1 (5.0)	3 (15.0)	16 (80.0)
Total	2 (3.1)	9 (13.5)	55 (83.4)

* squamous cell carcinoma

DISCUSSION

Hybridization of *Eco*R I-digested DNA blots from oral squamous cell carcinoma cell lines, HSC–2, –3 and Ca9–22 with the v-*fms* probe revealed a RFLP for this gene. The *Eco*R I restriction profiles observed in analysis of DNA samples of HSC–2, –3 and normal peripheral blood (WBC) were all identical. In the DNA isolated from Ca9–22, a 28.5kb *Eco*R I fragment appeared instead of the 12.5kb one. The 3.3kb *Bam*H I restriction fragment was shifted to 2.9kb. However, the *Hin*d III restriction profiles remained unchanged in these three lines. On the restriction enzyme map of the c-*fms* gene, the v-*fms* probe detected four fragments of 3.2, 3.2, 1.2 and 2.8kb. If about 400bp were deleted in one 3.2kb fragment, the *Hin*d III restriction fragments would be 2.8, 3.2, 1.2 and 2.8kb. Thus, we speculated that the 400bp were deleted in the 3.2kb *Hin*d III fragment. Therefore, we used the c-*fms* probe encoding the *Eco*R I-*Hin*d III fragment (0.9kb) of the human c-*fms* gene, which simplified the RFLP. *Eco*R I, *Bam*H I and *Hin*d III endonuclease digestion indicated that about the 400bp containing an *Eco*R I site was deleted in intron 11 and/or exon 12.

We cloned the deleting fragment by PCR because the human c-*fms* gene has already been cloned and sequenced by Hampe et al[5] Oliogonucleotides used for priming PCR were 20mers. The predicted PCR product of the normal gene was 582bp and that for the deleting fragment was about 180bp. The lengths that appeared on the ethidium bromide-stained gel from the 40 cycle reaction were 580, 490 and 160bp. We believe that the normal and deleting types were 580 and 160bp, respectively. More detailed information about the genetic organization of the region missing in Ca9–22 was obtained by DNA sequencing analysis. The size of the deletion is 426bp and the sequence of the start and end points was AAG. The 490bp DNA band revealed by PCR and ethidium bromide staining, was not predicted.

A RFLP in the c-*fms* gene has been previously identified in a random adult population[20,22]. Our sequencing data was the same as that reported by Verbeek[20], who studied a patient with acute lymphocytic leukemia.

In order to examine whether the deletion affected the expression of the c-*fms* allele was examined mRNA of Ca9–22 by Northern hybridization. Total cellular RNA was Northern blotted with a v-*fms* probe as described above. A 4.0kb transcript was detected in HSC–2, –3 and Ca9–22. However, the intensities were almost equal and no abnormal bands appeared. Artificial yeast introns that show cold-sensitive splicing have been constructed by Yoshimatsu et al[24]. These conditional introns can be inserted into a target gene as an "intron cassette" without dis-

rupting the coding information, allowing expression of the gene to be cold sensitive. The advantage of this intron-mediated control system is that a gene can be converted to a controllable gene by simple insertion of an intron. The molecular basis of the cholesteryl ester transfer protein (CETP) deficiency was investigated in 4 unreleted CETP-deficiency families by Yamashita et al[23]. Two patients were homozygous for a G-to-A change at the 5'–splice donor site of intron 14, causing impaired splicing of pre-mRNA. We predicted an abnormal transcript of the deleted c-*fms* gene, but no significant difference among these three cell lines was detected by Northern hybridization.

The c-*fms* oncogene product was a glycoprotein with tyrosine kinase activity[11,12,18]. We examined the c-*fms* product in Ca9–22 and other cell lines by means of an immune-complex kinase assay performed with polyclonal antibody to c-*fms*-coded epitopes. The antibody precipitated 150 and 130kDa proteins from Ca9–22 and HSC–2. The c-*fms* product is synthesized as an immature intracellular glycoprotein that has an apparent molecular mass of 130kDa. During intracellular transport to the plasma membrane, the *N*-linked carbohydrates undergo modification, resulting in an increase in the apparent molecular mass to 150kDa[17]. We obtained reasonable results but both the wild and deleting types had similar intensity and mobility.

Colony-stimulating factor–1 (CSF–1), also referred to as macrophage colony-stimulating factor (M-CSF) is a lineage-specific hematopoietin that stimulates proliferation and supports differentiation and survival of cells of the mononuclear phagocyte series[9]. Roussel et al[13] reported that mouse NIH3T3 cells expressing human colony-stimulating factor–1 (CSF–1) receptors had overgrown in serum-free medium containing human CSF–1 as their only growth factor. We examined the effect of the ligand CSF–1 upon Ca9–22 at various concentrations of recombinant human M-CSF, but Ca9–22 did not overgrow in the presence of 0.1% fetal bovine serum.

Boultwood et al[1] reported that the loss of the *CSF1R* (*FMS*) gene in patients with myelodysplasia might be important in the pathogenesis of human myeloid leukemia. Employing RFLP analysis and gene dosage studies, they found that all 10 patients had a deletion of *CSF1R*. We examined the frequency of c-*fms* alleles in oral cancer patients using RFLP analysis. *Eco*R I, *Bam*H I and *Hin*d III endonuclease digestion indicates the presence of two alleles, *a* and *b*. The distribution in the analyzed population (46 oral tumors and 20 peripheral leukocyte samples from oral cancer patients as well as normal adults) was 13.5% heterozygotic *ab*, 83.4% homozygotic *bb*, and 3.1% homozygotic *aa*. Xu et al[22] reported similar data that the distribution in 48 unrelated individuals was 23% *ab*, 75% *bb*, and 2% *aa*. We examined the loss of heterozygosity in the oral cancer patients. Compared with tumors and peripheral blood samples, the loss of heterozygosity was not detectable in all samples. The biological significance of the c-*fms* RFLP that caused the 426bp deletion in intron 11, has not yet been elucidated.

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