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Adhesiveness of β5 integrin variant lacking FNK$^{767\text{-}769}$ is similar to that of the prototype containing FNKFNK$^{764\text{-}769}$

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Abstract

Little is known about the functions of two different β5 integrin: repeated-FNK (FNKFNK$^{764\text{-}769}$) and single-FNK (FNK$^{764\text{-}766}$) amino acid sequence in the cytoplasmic domain. We examined whether it occurred as germ line mutation or somatic mutation associated with neoplastic transformation, and whether there is functional alteration. Among 6 culture cell lines only KATO-III cells have the single-FNK β5 sequence, while other cell lines do not. The single-FNK β5 was found in 9 of 79 patients with colon carcinoma, but no somatic mutations were detected in cancer tissues. CHO cells were transformed with expression vectors containing single-FNK or repeated-FNK β5 cDNA, which were derived from KATO-III cells. CHO cells transfected with single-FNK and with repeated-FNK showed similar adhesiveness to and proliferative activity on vitronectin substrates.

Keywords: β5 integrin; Adhesion; Vitronectin.

1. Introduction

The members of the integrin family are characterised by the ability to dynamically regulate their ligand binding affinity; integrin-mediated cell anchorage to the extracellular matrix has also been shown to induce signal transduction pathways (Hynes, 1992; Guadagno *et al.*, 1993; Juliano and Haskill, 1993). The cytoplasmic sequences of the integrins appear to be involved in signalling events (O'Toole *et al.*, 1994), and experimental studies have revealed a crucial role of the cytoplasmic domains in maintaining the proteins' functions (Stephens *et al.*, 1993). In addition, it was reported that alternative splicing variants of β subunits in α6, β1, β3 and αIIb usually occur in cytoplasmic domains, with many of the variants being functionally inactive.

The αvβ5 integrin is expressed by most epithelial cells (Pasqualini *et al.*, 1993). Its function differs from that of the major vitronectin receptor, αvβ3, and the cytoplasmic domain of β5 can transduce adhesion information to subsequent signalling pathways (Leavesly *et al.*, 1992; Pasqualini and Hemler 1994). The cDNA sequence of the β5 subunit from the UCLA-P3 human lung carcinoma cell line (McLean *et al.*, 1990), and that obtained from a thymic epithelial cDNA library (Ramaswamy and Hemler, 1990) have a repeated-FNK sequence ($FNKFNK^{764\text{-}769}$) in the cytoplasmic domain. Moreover, a different β5 sequence has been reported by Suzuki *et al.* (1990) in an uncharacterised cell line in which $FNK^{767\text{-}769}$ was deleted, resulting in a single FNK sequence. Although Ramaswamy and Hemler (1990) suggested that the repeated FNK sequence was the prototype of β5, they identified five of eight clones in a λgt11 thymic epithelial cDNA library that lacked the second FNK. In addition, other types of heterogeneity were found at positions 336-338, 379-381 and 708. These findings indicate molecular polymorphism of the β5 integrin subunit, whereas the cytoplasmic domains of other integrin subunits are highly conserved, except for alternative splicing. However, nothing is known about the significance of the polymorphism of β5.

In this report, we examined the incidence of the variant type β5 integrin containing single FNK sequence in using colon tissues, and examined whether it occurred as somatic mutation in carcinoma tissue. Furthermore, we tried to clarify whether it had functional alteration. Here, we report that adhesiveness and proliferative activity of the variant type, which appear in 10% of the population, are similar to those of the prototype β5 integrin.

2. Materials and Methods

2.1 Carcinoma cell lines

A variety of cell lines, including the oral squamous carcinoma lines OSC-19 and HSC-3 (Kawahara *et al.*, 1995), the lung carcinoma lines Calu-1, VMRC-LCP, ACC-LC-73 (Suzuki *et al.*, 1993) and LU65, and the gastric carcinoma cell line KATO-III (Sekiguchi, 1978), were used in the present study. Sub-confluent cultures were used to examine mRNA expression. All cell lines were maintained in Eagle's minimum essential medium containing 10% foetal bovine serum (Gibco BRL, Rockville, MD, USA). HSC-3, VMRC-LCP, LU65 and KATO-III were obtained from the Japanese Cancer Resource Bank, and ACC-LC-73 and Calu-1 were provided by Dr. T. Takahashi (Aichi Cancer Institute, Japan).

2.2 Reverse transcription (RT) - PCR

Total cellular RNA was extracted by the guanidinium isothiocyanate method (Chomozynski *et al.*, 1987) using a Total RNA Separator Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA). Poly (A+) mRNA was obtained from the cultured carcinoma cells by using an oligo-dT column (Poly (A) Quick Kit, Stratagene, La Jolla, CA, USA). First-strand cDNA was synthesised using AMV reverse transcriptase (Takara Bio, Ohtsu, Japan). The resulting cDNA was used for PCR amplification of an intracytoplasmic domain of β5 integrins, using primer pairs that amplify a 219-bp fragment encoding the cytoplasmic region of β5, *i.e.*, B5VNR5, 5'-^{2514}TGTGGTCGGTAGCATCCTCC^{2533}-3' and B5VNR3, 5'-^{2765}CCCGCTCCAGCCCCTCGGAG^{2746}-3', according to the sequence of Hemler *et al.*

(EMBL/GenBank/DDBJ, accession no. X53002). The PCR conditions were as follows: 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were electrophoresed on agarose gels, which were stained with ethidium bromide before or after electrophoresis. *Hae*III fragments of ϕX174 replicative form DNA were used as molecular size markers (Toyobo, Osaka, Japan).

2.3 PCR/Single-strand conformation polymorphism (SSCP) analysis

SSCP analysis was performed as described previously (Akasofu and Oda, 1995). DNA was subjected to PCR using a set of primers, B5VNR5 and B5VNR3, labelled with [γ-^{32}P] ATP (7,000 Ci/mmol, 160 mCi/ml, ICN, Costa Mesa, CA, USA) by the polynucleotide kinase reaction (Takara). The PCR products in formamide were heated to 80°C for 5 min, and then loaded onto gels. Polyacrylamide gel electrophoresis was performed at 40 watts for 3 h at 4°C. The gels were dried on filter paper and exposed to X-ray film at –80°C for 1-12 h with an intensifying screen.

2.4 S1 nuclease digestion

After electrophoresis, bands were cut out of the agarose gels and the DNA was extracted using filtration tubes (Suprec-01, Takara). The purified samples were incubated for 30 min at 37°C with 750 U of S1 nuclease (Takara) in 200 µl of S1 nuclease solution (30 mM sodium acetate, pH 4.6, 280 mM NaCl and 1 mM $ZnSO_4$). After ethanol precipitation, the samples were electrophoresed in 2% agarose gels, and then stained with ethidium bromide.

2.5 Direct sequencing

After PCR and electrophoresis on agarose gels, the bands were cut out and extracted using filtration tubes. The amplified strands were labelled with fluorescein by the dideoxynucleotide chain termination method using a DyeDeoxy Terminator Cycling Kit (Perkin-Elmer, Foster City, CA, USA). DNAs were analysed with an autosequencer and a 373 DNA sequence analyser (Perkin-Elmer).

2.6 TA cloning

The PCR products were subcloned into vectors by the TA cloning method (Clark, 1988) using a TA cloning kit (Invitrogen Corp., Carlsbad, CA, USA). Briefly, fresh PCR products amplified using Taq polymerase were ligated into linearised pCR2.1 (Invitrogen) using T4 DNA ligase. The ligated vectors were transformed into competent INVαF7 cells and grown overnight on LB agar plates containing ampicillin and X-gal. Blue-white screening was performed for inserts. The ligated clones were purified and the inserts were sequenced using the M13(-20) forward primer and a DyeDeoxy Terminator Cycling Kit.

2.7 Tissue samples

Tissue samples were obtained from 33 patients with colon carcinoma, who underwent surgery at Kanazawa University Hospital. Tumour and non-tumour tissues were dissected immediately after operation. Genomic DNA was extracted from the tissues by the standard procedure using a DNA extraction kit (Stratagene). Informed consent was obtained from all patients enrolled in the study.

2.8 Expression vector and transfection

β5 Integrin cDNA (repeated FNK) inserted into the expression vector pcDNA1.1Neo was kindly provided by Dr. D.A. Cheresh (The Scripps Research Institute, La Jolla, CA, USA). β5 cDNA was cut out of the vector with *Bam*HI and *Hin*dIII and reinserted into the permanent expression vector, pcDNA3.1 (Invitrogen). The resultant vector, FNKFNK/pCDNA3.1, was transfected into CHO-K1 cells by lipofection using Effectene Transfection Reagent (QIAGEN, Chatsworth, CA, USA). Transfected cells were cultured in Ham's F12 medium containing 10% foetal bovine serum and 400 μg/ml G418 (Sigma, St. Louis, MO, USA) for 1 week. Surviving cells were harvested and cloned on 96-well plates.

2.9 Immunoprecipitation

Cell-surface proteins were biotinylated with ECL Biotinylation Reagents (Amersham

Pharmacia Biotech, Piscataway, NJ, USA). Cell lysates obtained with cell lysis buffer (250 mM NaCl, 25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.5% NP-40, 1 mM PMSF, 2 μg/ml aprotinin and 1 μg/ml leupeptin) were centrifuged and the supernatants were reacted with 1 μg of anti-β5 integrin antibody (clone IA-IV5, provided by Dr. Hemler) and with 100 μl of protein G-Sepharose (Amersham Pharmacia Biotech) for 3 h at 4°C. After washing 6 times with cell lysis buffer, sodium dodecyl sulphate sample buffer (62.5 mM Tris-HCl buffer, pH 6.8, 2% sodium dodecyl sulphate, 10% glycerol, 0.1% bromophenol blue, 6% mercaptoethanol) was added to the gel, and then heated at 95°C for 5 min. Supernatants were subjected to 6% polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Perkin-Elmer). The membranes were reacted with peroxidase-conjugated streptavidin. Visualisation was performed using a chemiluminescence system (ECL Western Blotting Detection Reagent, Amersham Pharmacia Biotech).

2.10 *Adhesion assay*

Flat-bottomed polystyrene, 24-well plates (Falcon Products, Oxford, CA, USA) were coated with 10 μg/ml vitronectin purified from human plasma (Kawahara *et al.*, 1995) in phosphate-buffered saline overnight at 4°C. The wells were then blocked with 0.5% bovine serum albumin overnight at 4°C. Then, 50,000 cells in serum-free medium were loaded onto ligands and incubated in a CO_2 incubator in quadruplicate. Non-adherent cells were removed by gentle washing with serum-free medium five times at room temperature. The status of adherent and non-adherent cells after washing was checked by a light microscopy, and the remaining adherent cells were quantified by MTT assay (Mossman, 1983). After removal of non-adherent cells, the plates were filled with 200 μl medium and 30 μl 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma) solution (5 mg/ml in distilled water), then incubated for 3 hours at 37 ºC. After removing the solution the insoluble dye product was solubilized with 200 μl dimethylsulfoxide, and the absorbance at

492 nm was measured.

2.11 *Statistics*

Student's *t* test was used for comparison of the averages of two groups. Two-way analysis of variance was used for comparison of kinetic data between two groups.

3. Results

3.1 Heterogeneity of the cytoplasmic domain of the β5 subunit in Kato-III cell cDNA

First, we examined the expression of β5 mRNA by RT-PCR with the primer pair B5VNR5 and B5VNR3. All cells examined showed a single band at 219 bp when DNA was run on an agarose gel containing ethidium bromide (Fig. 1A). Amplified products from all cells were sequenced directly. The deduced amino acid sequence of β5 at positions 764 to 769 in seven of the eight carcinoma cell lines examined was $FNKFNK^{764\text{-}769}$. This sequence, reported previously by McLean *et al.* (1990) and Ramaswamy and Hemler (1990), is referred to as the prototype sequence. The sequence pattern of the PCR-amplified β5 of KATO-III showed a double peak downstream of nucleotide 2,553 using the sense primer (Fig. 1B), and upstream of nucleotide 2,562 using the antisense primer, indicating the presence of multiple components with the same sequence between position 2,553 and 2,562. This pattern was considered to reflect the presence of a species with a 9-base deletion at base 32 upstream from the stop codon, which is consistent with the sequence reported by Suzuki *et al.* (1990). The 9-base deletion results in deletion of the amino acid sequence $FNK^{767\text{-}769}$. To confirm the presence of the two components, we performed SSCP analysis. β5 cDNAs from KATO-III, LU65 and Calu-1 cells were amplified by PCR and electrophoresed. The PCR products of KATO-III showed four bands, two of which were at the same positions as those of Calu-1 and LU65 cells (Fig. 1C), indicating the presence of a mixture of PCR products with two different sequences. Subsequently, we subcloned the PCR products with a TA cloning system. One

colony showed the prototype sequence (Fig. 1D, top), and another showed the 9-base deletion (Fig. 1D, bottom).

3.2 Double bands appeared in heterozygous expression

Furthermore, we found that the PCR products of KATO-III cDNA had double bands around 219 bp that were separated by more than the size of the 9-bp deletion on agarose gels stained with ethidium bromide after electrophoresis (Fig. 2A). All cell lines except KATO-III showed single bands. The difference between the double bands seemed to be larger than 9 bp, and the DNAs extracted from the upper and lower bands were sequenced separately. Surprisingly, they had the same sequence patterns, showing that they contained mixtures of DNA (Fig. 2B). To address this problem, we considered a mismatch and match hypothesis. That is, both the upper and lower bands were composed of a mixture of the prototype and the deletion type, but the DNA of the upper band was composed of only double-stranded mismatched DNA whose secondary structure formed a loop, resulting in a lower electrophoretic mobility. On the other hand, the lower bands were composed of non-mismatched double-stranded DNA of prototype and mutant type that showed no mobility differences under the electrophoretic conditions used. To test this hypothesis, S1 nuclease was used to digest single-stranded, but not double-stranded, DNA, as occurs in the non-hybridised portions of a loop in mismatched DNA. After S1 nuclease digestion, the upper band showed a shift in mobility to the same position as the lower band, while the position of the lower band was not altered (Fig. 2C).

3.3 Intron sequence of the cytoplasmic domain of β5

We investigated whether the 9-base deletion in the cDNA was caused by a deletion in the genomic DNA, alternative splicing or mutation at the exon-intron boundary. For this purpose, it was necessary to analyse the genomic DNA sequence and to establish the location and sequence of the intron in the cytoplasmic domain of β5. We assumed that all α and β

integrin subunits have introns at similar positions (McLean *et al.*, 1990), and hence we examined the intron position at a location corresponding to that of β3 and αIIb (van Kuppevelt *et al.*, 1989; Prandini *et al.*, 1988), near the sequence encoding FNK. Genomic DNA of Calu-1 cells was amplified using primers B5VNR5 and B5VNR3, which amplify a cDNA fragment of 219 bp. The PCR product contained about 890 bp, of which 670 bp were assumed to be the intron. The PCR products were sequenced and we determined a partial sequence of the intron. The results indicated that the intron was located 55 bp upstream of the first nucleotide corresponding to FNK. We prepared a newly designed primer spanning the intron sequence, B5INT1U, 5'-ACTGTGTCCTCATCTCTTCC-3', and used it, together with the antisense primer B5VNR3, to amplify the variable sites of the genomic DNA (Fig. 3). With the exception of KATO-III, the PCR products of 6 carcinoma cell lines showed single bands at 159 bp. The PCR products of KATO-III appeared as two bands separated by more than 9 bp under the same conditions (data not shown). The PCR products of KATO-III were subcloned into pCR2.1. One clone had the same sequence as Calu-1 (repeated FNK), and another clone had a 9-base deletion in the exon sequence (single FNK).

3.4 Prevalence of the single-FNK β5 in cancer and non-cancer tissues

It is not known whether the 9-base deletion represents a somatic mutation occurring in the course of malignant transformation or a germ line mutation. Somatic mutation seems likely, because the single FNK sequence detected by Suzuki *et al.* (1990) and by us was present in carcinoma cells. On the other hand, Ramaswamy and Hemler (1990) identified five of eight clones in a λgt11 thymic epithelial cDNA library that lacked the second FNK. Hence, the protein with a single FNK may be a genetically determined variant of the β5 subunit. The observation that heterogeneous genomic DNA, including partial deletion, led to two fully separated electrophoretic bands provides a powerful means for detecting the heterozygous β5 gene. Genomic DNA was extracted from colon cancer tissues from 89 patients, and the β5

integrin gene encoding the cytoplasmic domain was amplified with B5INT1U and B5VNR3. Seventy-nine cases showed a single band migrating to a position corresponding to 159 bp on agarose gel electrophoreses (homozygous), while double bands around this position were detected in 9 cases (11%) that were considered heterozygous in the β5 integrin gene (Fig. 4A). We further examined genomic DNA of non-cancer tissues. Heterozygous β5 in non-cancer tissues was only detected in the cases in which cancer tissues were also heterozygous (Fig. 4B), indicating the absence of somatic mutation associated with neoplastic transformation.

3.5 Transformation of CHO cell by single-FNK β5 and repeated-FNK β5

As the function of β5 has been examined using repeated-FNK β5 (Pasqualini *et al*., 1993; Pasqualini and Hemler, 1994), it is necessary to determine whether single-FNK β5 has the same function as repeated-FNK β5. To address this question, single-FNK β5 and repeated-FNK β5 were transfected into CHO cells. To obtain a full-length single-FNK β5 cDNA, a portion of β5 cDNA of KATO-III containing the sequence corresponding to the single FNK subcloned into pCR2.1 was used. The subcloned DNA containing the single FNK sequence was truncated with *Bsp*HI and *Xba*I. The portion of cDNA containing repeated-FNK was cut out of the expression vector FNKFNK/pcDNA3.1 by digestion with the same restriction enzymes and the single-FNK sequence was reinserted (FNK/pcDNA3.1). FNK/pcDNA3.1 and FNKFNK/pcDNA3.1 were transfected into CHO cells, and clones were selected. To confirm transfection of FNK/pcDNA3.1 and FNKFNK/pcDNA3.1, genomic DNA and RNA were extracted from the transfected CHO cells. PCR using primers to amplify β5 cDNA showed corresponding bands in CHO cells transfected with FNKFNK/pcDNA3.1 and with FNK/pcDNA3.1, but not in non-transfected CHO cells (Fig. 5A). RT-PCR using primers that amplify β5 cDNA also showed the corresponding bands in transfected CHO cells (Fig. 5B). Surface expression of β5 in the transformed cells was confirmed by immunoprecipitation using biotinylated cell-surface proteins (Fig. 5C). To determine the adhesiveness of the

transformants to vitronectin, the kinetics of adhesion of CHO cells transfected with FNKFNK/pcDNA3.1 and with FNK/pcDNA3.1 were examined. The results showed partial adhesion of the cells to vitronectin at 10, 20 and 30 min, and no significant difference was detected in adhesiveness (Fig. 6A). We also examined proliferative activity on vitronectin for 5 days. Cells suspended in serum-free medium were loaded onto vitronectin-coated dishes, and incubated for 5 days in a CO_2 incubator at 37°C. Cells were harvested with 0.25% trypsin/EDTA, and counted with a hemocytometer under a light microscope. There was no significant difference between FNK-transfected cells and FNKFNK-transfected cells (Fig. 6B).

4. Discussion

KATO-III cells were confirmed to contain two distinct β5 sequences, as described previously (McLean *et al.*, 1990; Ramaswamy and Hemler, 1990; Suzuki *et al.*, 1990). However, no information is available about the significance of this polymorphism of β5. First, we investigated whether deletion of one FNK sequence in cDNA was caused by deletion in the genomic DNA, alternative splicing or mutation at the exon-intron boundary. Our results indicated that the polymorphism is due to a 9-base deletion in the genomic DNA. The single-FNK β5 was concluded to be a genetically determined variant of the prototype β5 subunit. This variant type was shown to be expressed as a cell-surface protein, which might associate with αv integrin subunit to form a heterodimer. We further found that cells bearing the variant had similar characteristics with regard to adhesiveness and proliferation to cells bearing the prototype β5.

The variant differs in the cytoplasmic domain, which is the functionally crucial portion of integrin. The cytoplasmic sequences of the integrins are involved in the bi-directional signalling events (O'Toole *et al.*, 1994; Hynes *et al.*, 2002). Src kinase

constitutively binds to the cytoplasmic domain of integrin β3 and the binding of integrin to extracellular ligands triggers outside-in signals (Arias-Salgado *et al.*, 2003). The cytoplasmic domain of integrin also regulates inside-out signalling. Conformational change of extracellular domain is induced by the interaction of α and β at those cytoplasmic domains (Takagi *et al.*, 2002), and the talin can directly activate integrins by binding to the β subunit at the cytoplasmic domain (Vinogradova *et al.*, 2002). Patients with Glanzmann thrombasthenia were detected in β3 subunit of the cytoplasmic domain (Chen *et al.*, 1992) and mutation of β2 in th cytoplamic domain causes leukocyte adhesion deficiency (Anderson *et al.*, 1987). Experimental study showed that deletion or substitution of one amino acid results in loss of integrin function (Reszka *et al.*, 1992; Stephens *et al.*, 1996; Languino and Ruoslahti, 1992; Meredith *et al.*, 1995), and conversely activates integrin function constitutively (Hughes *et al.*, 1996). In addition, it has been reported that alternative splicing variants of β subunits, which were functionally inactive, occur in the cytoplasmic domains. Thus, a single amino acid substitution in the cytoplasmic domain can cause loss of function, and the variant type lacks three amino acids. Therefore, we initially considered that loss of function was likely. However, this was not the case. Studies of the cytoplasmic domain of avian β1 (Reszka *et al.*, 1992) identified three potentially important regions, designated cyto-1, cyto-2 and cyto-3, with the sequences HDRREFAKFEKE, NPIY and NPKY, respectively. These sequences are conserved in the β1, β2, β3, β5, β6 and β7 integrin subunits. When β1D, a variant of β1A that contains cyto-2 and cyto-3, was transfected into CHO cells, no alterations were observed in adhesiveness or tyrosine phosphorylation (Belkin *et al.*, 1996), whereas β1B and β1C, which lack cyto-2 and cyto-3, were shown to be functionally inactive (Languino and Ruoslahti, 1992; Meredith *et al.*, 1995). Furthermore, cyto-2 and/or cyto-3 have been shown to be important for adhesiveness (Reszka *et al.*, 1992; O'Toole *et al.*, 1995). The NKSY sequence of β5 integrin is a motif corresponding to cyto-3 at six residues upstream from the N-terminus.

The deletion of one FNK converts FNKFNKSYN to FNKSYN, thus conserving the cyto-3 sequence. This may be why the single-FNK β5 variant retains full functionality.

References

AKASOFU M, ODA Y, 1995. Immunohistochemical detection of p53 in cervical epithelial lesions with or without infection of human papilloma virus types 16 and 18. VIRCHOWS ARCH 425: 593-602.

ANDERSON DC, SPRINGER TA, 1987. Leukocyte adhesion deficiency: an inherited defect in the Mac-1, LFA-1, and p150, 95 glycoproteins. ANNU REV MED. 38: 175-94.

ARIAS-SALGADO EG, LIZANO S, SARKAR S, BRUGGE JS, GINSBERG MH, SHATTIL SJ, 2003. Src kinase activation by direct interaction with the integrin β cytoplasmic domain. PROC NATL ACAD SCI USA 100: 13298-13302.

BELKIN AM, ZHIDKOVA NI, BALZAC F, ALTRUDA F, TOMATIS D, MAIER A, TARONE G, KOTELIASNSKY VE, BURRIDGE K, 1996. β1D integrin displaces the β1A isoform in striated muscles. Localization at junctional structures and signaling potential in nonmuscle cells. J CELL BIOL 132: 211-226.

CHEN Y-P, DIAFFAR I, PIDARD D, STEINER B, CIEUTAT A-M, CAEN JP, ROSA J-P, 1992. Ser-752-Pro mutation in the cytoplasmic domain of integrin β3 subunit and defective activation of platelet integrin αIIbβ3 (glycoprotein IIb-IIIa) in a variant of Glanzmann thrombasthenia. PROC NATL ACAD SCI USA, 89: 10169-10173.

CHOMOZYNSKI P, SACCHI N, 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. ANAL BIOCHEM 162: 156-159.

CLARK JM, 1988. Novel non-templated nucleotide addition reactions catalyzed by prokaryotic and eukaryotic DNA polymerase. NUCL ACIDS RES 16: 9677-9686.

GUADAGNO TM, OHTSUBO M, ROBERTS JM, ASSOIAN RK, 1993. A link between cyclin A expression and adhesion-dependent cell cycle progression. SCIENCE 262: 1572-1575.

HUGHES PE, DIAZ-GONZALEZ F, LEONG L, WU C, MCDONALD JA, SHATTIL SJ,

GINSBERG MH, 1996. Breaking the integrin hinge. A defined structural constraint regulates integrin signaling. J BIOL CHEM 271: 6571-6574.

HYNES RO, 1992. Integrins: Versatility modulation and signaling in cell adhesion. CELL 69: 11-25.

HYNES RO, 2002. Integrins: Bidirectional, allosteric signaling machines. CELL 110: 673-687.

JULIANO RL, HASKILL S, 1993. Signal transduction from the extracellular matrix. J CELL BIOL 120: 577-585.

KAWAHARA E, IMAI K, KUMAGAI S, YAMAMOTO E, NAKANISHI I, 1995. Inhibitory effects of adhesion oligopeptides on the invasion of squamous cell carcinoma cells with special reference to implication of αv integrins. J CANCER RES CLIN ONCOL 121: 133-140.

KAWAHARA E, NAKADA N, HIKICHI T, KOBAYASHI J, NAKANISHI I, 2002. EGF and β1 integrin convergently regulate migration of A431 carcinoma cell through MAP kinase activation. EXP CELL RES 72: 84-91.

LANGUINO LR, RUOSLAHTI E, 1992. An alternative form of the integrin β1 subunit with a variant cytoplasmic domain. J BIOL CHEM 267: 7116-7120.

LEAVESLY DI, SCHWARTZ MA, ROSENFELD M, CHERESH DA, 1992. Requirement of the integrin β3 subunit for carcinoma cell spreading or migration on vitronectin and fibrinogen. J CELL BIOL 117: 1101-1107.

MCLEAN JW, VESTAL DJ, CHERESH DA, BODARY SC, 1990. cDNA sequence of the human integrin β5 subunit. J BIOL CHEM 265: 17126-17131.

MEREDITH J JR, TAKADA Y, FORNARO M, LANGUINO LR, SCHWARTZ MA, 1995. Inhibition of cell cycle progression by the alternative spliced integrin β1C. SCIENCE 269: 1570-1572.

MOSSMAN T, 1983. Rapid colorimetric assay for cellular growth and survival. Application to proliferation and cytotoxicity assays. J IMMUNOL METHODS 65: 55-63.

O'TOOLE TE, KATAGIRI Y, FAULL RJ, PETER K, TAMURA R, QUARANTA V, LOFTUS JL, SHATTIL SJ, GINSBERG MH, 1994. Integrin cytoplasmic domains mediate inside-out signal transduction. J CELL BIOL 124: 1047-1059.

O'TOOLE TE, YLANNE J, CULLEY BM, 1995. Regulation of integrin affinity states through an NPXY motif in the β subunit cytoplasmic domain. J BIOL CHEM 270: 8553-8558.

PASQUALINI R, BODOROVA J, SONG Y, HEMLER ME, 1993. A study of the structure function and distribution of β5 integrins using novel anti-β5 monoclonal antibodies. J CELL SCI 105: 101-111.

PASQUALINI R, HEMLER ME, 1994. Contrasting roles for integrin β1 and β5 cytoplasmic domains in subcellular localization cell proliferation and cell migration. J CELL BIOL 125: 447-460.

PRANDINI MH, DENARIER E, FRACHET P, UZAN G, MARGUERIE G, 1988. Isolation of the human platelet glycoprotein IIb gene and characterization of the 5' flanking region. BIOCHEM BIOPHYS RES COMMUN 156: 595-601.

RAMASWAMY H, HEMLER ME, 1990. Cloning primary structure and properties of a novel human integrin β subunit. EMBO J 9: 1561-1568.

RESZKA AA, HAYASHI Y, HORWITZ AF, 1992. Identification of amino acid sequences in the integrin β1 cytoplasmic domain implicated in cytoskeletal association. J CELL BIOL 117: 1321-1330.

SEKIGUCHI M, SAKAKIBARA K, FUJII G, 1978. Establishment of cultured cell lines derived from a human gastric carcinoma. JPN J EXP MED 48: 61-68.

STEPHENS LE, SONNE JE, FITZGERALD ML, DAMSKY CH, 1993. Targeted deletion of

β1 integrins in F9 embryonal carcinoma cells affects morphological differentiation but not tissue-specific gene expression. J CELL BIOL 123: 1607-1620.

SUZUKI S, HUANG ZS, TANIHARA H, 1990. Cloning of an integrin β subunit exhibiting high homology with integrin β3 subunit. PROC NATL ACAD SCI USA 87: 5354-5358.

SUZUKI S, TAKAHASHI T, NAKAMURA S, KOIKE K, ARIYOSHI Y, TAKAHASHI T, UEDA R, 1993. Alterations of integrin expression in human lung cancer. JPN J CANCER RES 84: 168-174.

TAKAGI J, PETRE BM, WALZ T, SPRINGER TA, 2002.Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. Cell 110: 599-611.

VAN KUPPEVELT THMSM, LANGUINO LR, GAILIT JO, SUZUKI S, RUOSLAHTI E, 1989. An alternative cytoplasmic domain of the integrin β3 subunit. PROC NATL ACAD SCI USA 86: 5415-5418.

VINOGRADOVA O, VELYVIS A, VELYVIENE A, HU B, HAAS T, PLOW E, QIN J, 2002. A structural mechanism of integrin αIIbβ3 "inside-out" activation as regulated by its cytoplasmic face. CELL 110: 587-97.

Figure legends

Fig. 1. Heterozygous β5 in KATO-III. A. RT-PCR using primers that amplify the cytoplasmic domain of β5. The PCR products were run on 2% agarose gels containing 0.1% ethidium bromide. The PCR product was seen as a single band of around 219 bp. B. Electrophoretograms of PCR products of KATO-III. Direct sequencing was performed by the dideoxynucleotide chain termination method. Double peaks appeared after the CAA sequence (indicated by arrows), which suggested the presence of a mixture of FNKFNK β5 and FNK β5 with a deletion of 9 bp, TTCAACAAA. C. Single-strand conformational polymorphism analysis of heterozygous amplified DNA. Four bands were detected in the PCR product of β5 of KATO-III cells, whereas only two bands were found for Calu-1 and LU65 cells. D. The PCR product was subcloned using the TA-cloning method. One clone showed the sequence of FNK β5, and another showed the sequence of FNKFNK β5.

Fig. 2. Analysis of heterozygous β5 showing double bands. A. RT-PCR using primers that amplify the cytoplasmic domain of β5. After electrophoresis, the agarose gel was stained with ethidium bromide solution. The PCR product of KATO-III was seen as double bands around 219 bp, with an apparent difference of 20-30 bp. B. Electrophoretograms of PCR products of KATO-III extracted from the agarose gel. PCR products were extracted from the upper and lower bands, and were sequenced by the dideoxynucleotide chain termination method. Both electrophoretograms showed double peaks after the CAA sequence, marked by arrows, which indicated a mixture of FNKFNK β5 and FNK β5. C. The mismatch-match hypothesis was tested using S1 nuclease, which digests single-stranded DNA. DNAs were extracted from the upper and lower bands of the KATO-III double bands (Mix). After S1 nuclease treatment, the DNA of the upper band showed an electrophoretic mobility shift to the position of the lower band (UP). The position of the lower band did not change after S1 nuclease digestion (LW). NT, PCR product of KATO-III β5; M, molecular size standard (ϕ174 *Hae*III fragments). D

and E. Biophysical analysis of the mismatch-match hypothesis. DNAs were extracted from the upper band (UP1) and lower band (LW1) of KATO-III without denaturation. Both DNAs were denatured by heating to 96°C, and cooled rapidly (UP2, LW2) or gradually to allow renaturation (UP3, LW3). Absorbance at 260 nm was determined (D), and the DNAs were subjected to 2% agarose gel electrophoresis (E).

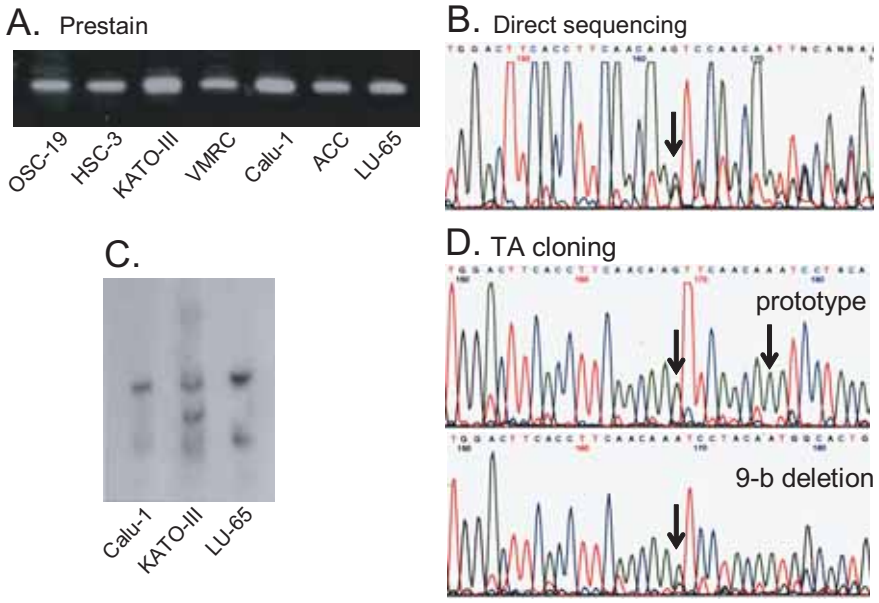
Fig. 3. Partial sequence of the intron in the cytoplasmic domain of β5 in Calu-1 cells. Primers that amplified the FNKFNK (bold) sequence of genomic DNA are underlined. The sequence deleted in FNK β5, AGTTCAACA, is boxed.

Fig. 4. Heterozygous β5 in cancer and non-cancer tissues. Cancer tissues (cases 11-19) and the corresponding non-cancer tissues were examined to determine the prevalence of FNK β5. PCR analysis with the primer pair that amplified genomic DNA containing the sequence encoding FNKFNK showed double bands in both the cancer (A) and non-cancer tissues from case 15 (B), indicating heterozygosity in β5 integrin. The markers were PCR products produced with the same primers using pCR2.1 with the FNKFNK β5 insert (M1) and pCR2.1 with the FNK β5 insert (M2) as the template.

Fig. 5. Characterisation of CHO cells transfected with FNK β5 and FNKFNK β5. Genomic DNA (A) and RNA (B) were extracted from non-transfected CHO (lanes 1), FNKFNK β5/CHO (lanes 2), and FNK β5/CHO (lanes 3). DNA or RNA was amplified by PCR or RT-PCR using primers that amplify the FNK portion. M, molecular size standard (ϕ174 *Hae*III fragments). (C) Immunoprecipitation using anti-β5 antibody from cells in which surface protein was labelled with biotin. Immunoprecipitated protein from non-transfected CHO (lane 1), FNKFNK β5/CHO (lane 2) and FNK β5/CHO (lane 3) was electrophoresed, transferred onto a PVDF membrane, reacted with streptavidin-HRP, and visualised by chemiluminescence. M, molecular size marker.

Fig. 6. Adhesiveness and proliferation of FNKFNK β5/CHO cells and FNK β5/CHO cells. (A) FNKFNK β5/CHO cells and FNKβ5/CHO cells were loaded onto vitronectin-coated 24-well dishes. Non-adherent cells were removed by gentle washing at 10, 20 and 30 min and the remaining adherent cells were quantified by MTT assay. The kinetics of adhesion of FNKFNK β5/CHO cells were compared with those of FNKβ5/CHO cells using two-way analysis of variance ($P>0.05$). (B) FNKβ5/CHO cells and FNKFNKβ5/CHO cells were loaded onto vitronectin-coated dishes 3.5 cm in diameter, and cultured for 5 days in a CO_2 incubator. Cells were dispersed with trypsin, and counted using a haemocytometer. Each bar shows the mean ± standard deviation of quadruplicate determinations. Student's *t* test was used for comparison of the averages of two groups ($P>0.05$).

Fig. 1.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OSC-19
HSC-3
KATO-III
VMRC
Calu-1
ACC
LU-65
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C.
Calu-1
KATO-III
LU-65
D. TA cloning
prototype
9-b deletion

Fig. 2.

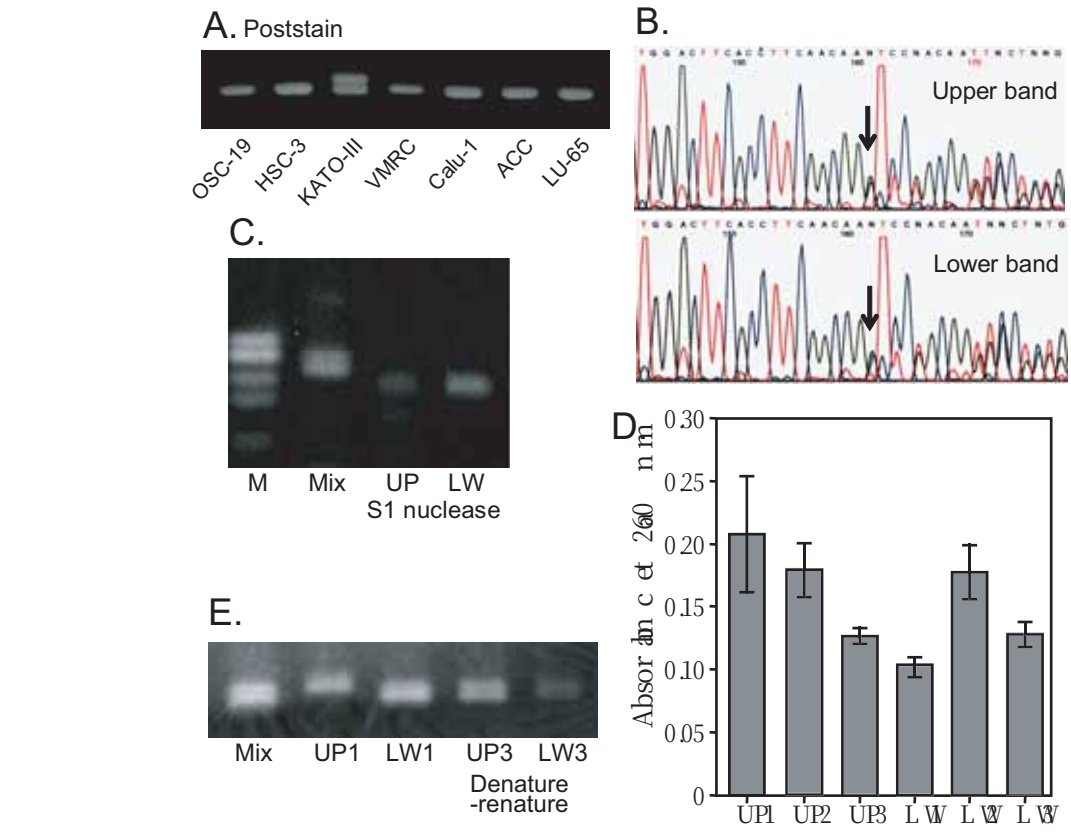
A. Poststain
OSC-19
HSC-3
KATO-III
VMRC
Calu-1
ACC
LU-65
B.
Upper band
Lower band
C.
M
Mix
UP
LW
S1 nuclease
D.
Absorbance at 260 nm
0.30
0.25
0.20
0.15
0.10
0.05
0
UP1
UP2
UP3
LW1
LW2
LW3
E.
Mix
UP1
LW1
UP3
LW3
Denature
-renature

Fig. 3.

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AGTTTGCAAA GTTTCAGAGC GAGCGATCCA GGGCCCGCTA TGAAATGGTA AGCACGTGGG AAATGGGAAG CAGAGGAGAC
E  F  A  K   F  Q  S   E  R  S   R  A  R  Y   E  M
TTCAAGCTCA GAGGCGTGGT TGAGTTCAGC GGTATGTTAG CAATAGAGGC CACTAATGTC TTTGGCATTA ----------
------------------TT TGAGTGTGTG AGCTAACACT GTGTCCTCAT CTCTTCCCCG CCGTGTTCTG TAGGCTTCAA
                                                 B5INT1U                          A  S
ATCCATTATA CAGAAAGCCT ATCTCCACGC ACACTGTGGA CTTCACCTTC AACAAGTTCA ACAAATCCTA CAATGGCACT
N  P  L  Y   R  K  P   I  S  T   H  T  V  D   F  T  F   N  K  F   N  K  S  Y   N  G  T
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V  D  J                B5VNR3
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Fig. 4.

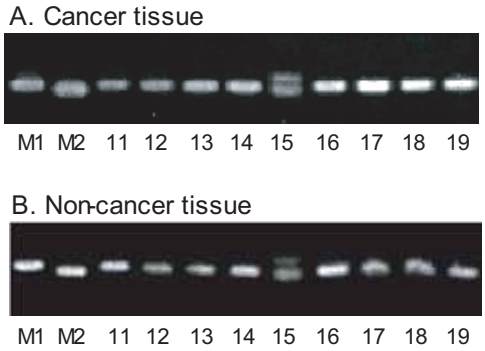

Fig. 5.

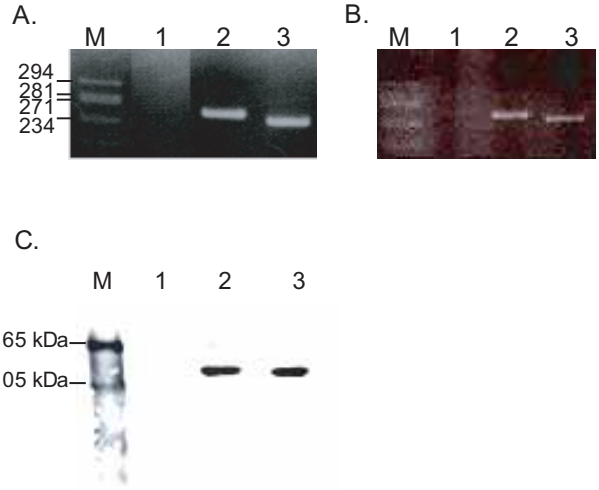

Fig. 6.