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A Deletion Mutation within the Ligand Binding Domain Is Responsible for Activation of Epidermal Growth Factor Receptor Gene in Human Brain Tumors

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Two transplantable cell lines of human glioblastoma multiforme GL-3 and GL-5 carried an amplification and overexpression of structurally altered epidermal growth factor (EGF) receptor gene: the 140 kilodalton EGF receptors in these cases exhibited a constitutively expressed tyrosine kinase activity without the ligand. Here, we isolated the abnormal EGF receptor cDNA from GL-5 cell line, and demonstrated that this cDNA bears a single large intramolecular deletion mutation 801 base pairs long within the ligand binding domain of EGF receptor. In other regions no amino acid substitution was observed. At the level of genomic DNA, this deletion appeared to start from the 1st intron and terminate in the 6th intron of the EGF receptor gene. However, in the two lines of glioblastoma, GL-3 and GL-5, the positions of the start or the end of the deletion mutation in these introns were not identical, suggesting an involvement of a unique recombination mechanism in the formation of deletion mutation. A weak but ligand-independent transforming activity was observed in the deletion-carrying EGF receptor cDNA.

Key words: Oncogene — Epidermal growth factor receptor — Deletion mutation — Glioblastoma multiforme

Glioblastoma multiforme, astrocytoma grade III and IV, is the major malignant tumor of human brain. Recent studies on oncogenes and anti-oncogenes have facilitated a survey of activated cellular genes in brain tumors which might be involved in the onset or progression of these malignancies. So far several proto-oncogenes have been found to be activated. Epidermal growth factor (EGF) receptor gene (c-*erbB* gene) was amplified in about one-third of the cases of glioblastoma multiforme.[1] Furthermore, most cases of glioblastoma multiforme bearing amplification of EGF-receptor gene have been reported to overexpress EGF receptor molecule.[2] Other oncogenes or possible oncogenes, c-*myc*,[3] N-*myc*,[4] *gli*[5] and *ros*-1[6] genes, were also observed to be amplified or rearranged in these tumors. However, activation of the latter four genes in glioblastoma is thought to be rare. Thus, the activation of proto-oncogenes at the DNA level is still unclear in more than half of the glioblastoma multiforme in humans. Further, although EGF receptor gene was found to be frequently amplified in these tumors, it has not been well studied yet whether these amplified genes are changed structurally and functionally.

In our recent report we have described that two out of 7 cases of human glioblastoma multiforme GL-3 and GL-5 examined carried amplification of structurally altered EGF receptor genes.[7] The transcripts and the products of these mutated EGF receptor genes were shorter than those expressed in normal tissue, and the 140 kilodalton (kDa) abnormal EGF receptors expressed in GL-3 and GL-5 showed a constitutively activated tyrosine kinase, independent of the ligand, similar to the products of v-*erbB* gene and the activated c-*erbB* gene formed through retrovirus insertion in chicken erythroblastosis.[8–10] Humphrey *et al.* also reportd several cases of human glioblastoma carrying amplification of structurally altered EGF receptor gene.[11] To analyze the primary structure and the transforming activity of the altered EGF receptor gene in human brain tumors and to see which mechanisms might be involved in this mutation, we have molecularly cloned the EGF receptor cDNA from GL-5 and its genomic DNA from GL-3 and GL-5 glioblastomas. Here we show that the alteration of the EGF receptor gene is due to a single large deletion within the ligand binding domain, and the mutated EGF receptor gene has a weak but ligand-independent transforming activity on NIH/3T3 cells.

MATERIALS AND METHODS

Screening of cDNA library GL-5 cells were maintained in athymic nude mice by transplantation of tumor pieces. Total RNA was isolated from fresh frozen GL-5 cells, and poly(A)$^+$RNA was purified by affinity chromatography using oligo-d(T) cellulose. A complementary DNA library was constructed in λgt11 via the unique *Eco*RI site and screened by plaque hybridization with a cloned EGF receptor cDNA, pE7, as a probe.[12]

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Screening of subgenomic library High-molecular-weight DNA, prepared from GL-5, GL-3 and placenta cells, was completely digested with *Hin*dIII and electrophoretically size-fractionated. Fractions containing the rearranged DNA fragments were purified and constructed in λ2001. The λ2001 subgenomic libraries were screened with ^{32}P-labeled 0.7kb *Ava*I-*Ava*I fragment of clone pE7.[12]

DNA sequencing The nucleotide sequence was determined by the dideoxy nucleotide chain-termination method.[13]

Construction of expression vectors Expression vectors containing the entire coding region of GL-5 EGF receptor cDNA were constructed as follows; a plasmid containing GL-5 cDNA was digested with *Sac*I and self-ligated (named p163). *Sac*I-*Bam*HI fragment of p163 was replaced by *Sac*I-*Bam*HI fragment of pE15. *Eco*RI-*Eco*RI fragments of p59-51 (#2317 *Eco*RI to the 3′-terminal *Eco*RI fragment) were ligated into the *Eco*RI site of these plasmids. The *Hin*dIII-*Sca*I fragment was isolated from these plasmids. The ends were filled in with Klenow enzyme, then methylated by *Bam*HI methylase, and *Bam*HI linkers were added for insertion into the *Bam*HI sites of the expression vectors pZIPneo-SV(X)I.[14]

DNA transfection assay DNA transfer into NIH3T3 cells was done by a polycation and dimethyl sulfoxide method as described elsewhere.[15] The transfected cells were grown in DMEM supplemented with 6% fetal or newborn calf serum in the presence or absence of 400 μg/ml of G418.

RESULTS AND DISCUSSION

Structure of EGF receptor cDNA obtaind from glioblastoma GL-5 Previously we showed that both GL-3 and GL-5 glioblastoma cell lines exhibit 20- to 30-fold amplification of the structurally altered EGF receptor gene.[7] Furthermore, Southern blotting analysis of GL-3 and GL-5 strongly suggested a large deletion mutation(s) within the ligand binding domain at the level of genomic DNA.[7] A cDNA library was prepared from glioblastoma GL-5 and screened with a fragment of EGF receptor cDNA as a probe (see "Materials and Methods"). The #59 clone which appeared to cover the whole coding

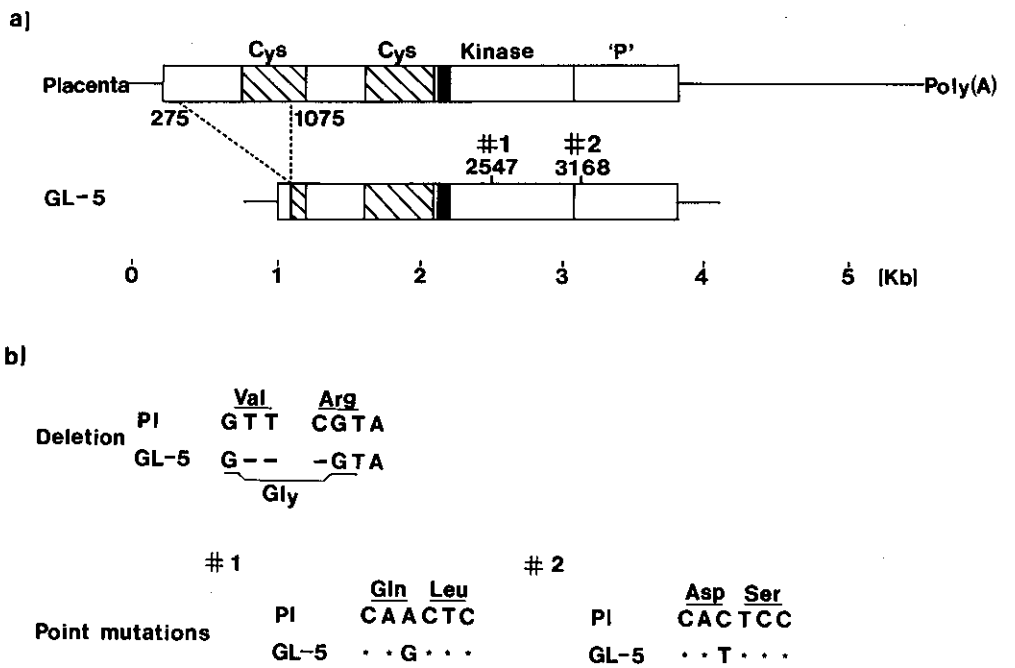


Fig. 1. Deletion mutation within the ligand binding domain of EGF receptor gene in glioblastoma multiforme GL-5. a) Schematic representation of the EGF receptor cDNA cloned from GL-5 glioblastoma cells. The deleted region extends from nucleotide residue 275 (within the codon for amino acid residue 30) to nucleotide residue 1075 (within the codon for amino acid residue 297).[16] The nucleotide numbers 2547 and 3168 indicate point mutations. Cysteine-rich regions, the tyrosine kinase domain and the autophosphorylation region are designated as Cys, Kinase and 'P', respectively. The solid box represents the transmembrane domain. b) An amino acid change at the junction of the deletion mutation and silent base changes in the GL-5 EGF receptor cDNA.

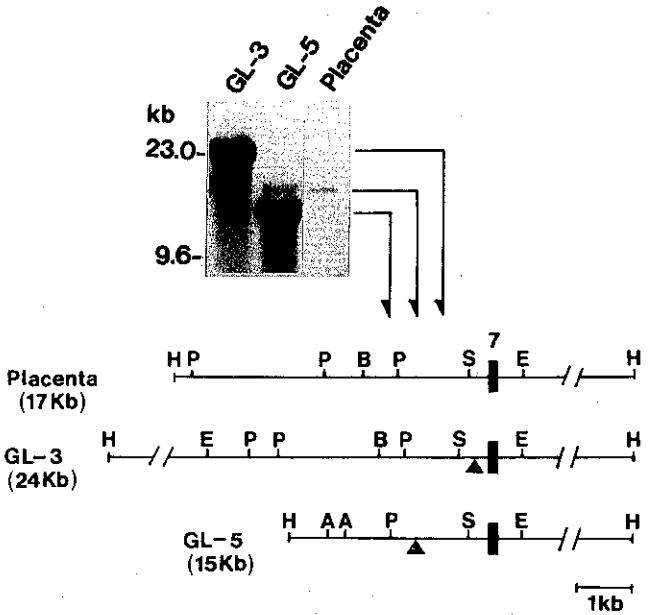
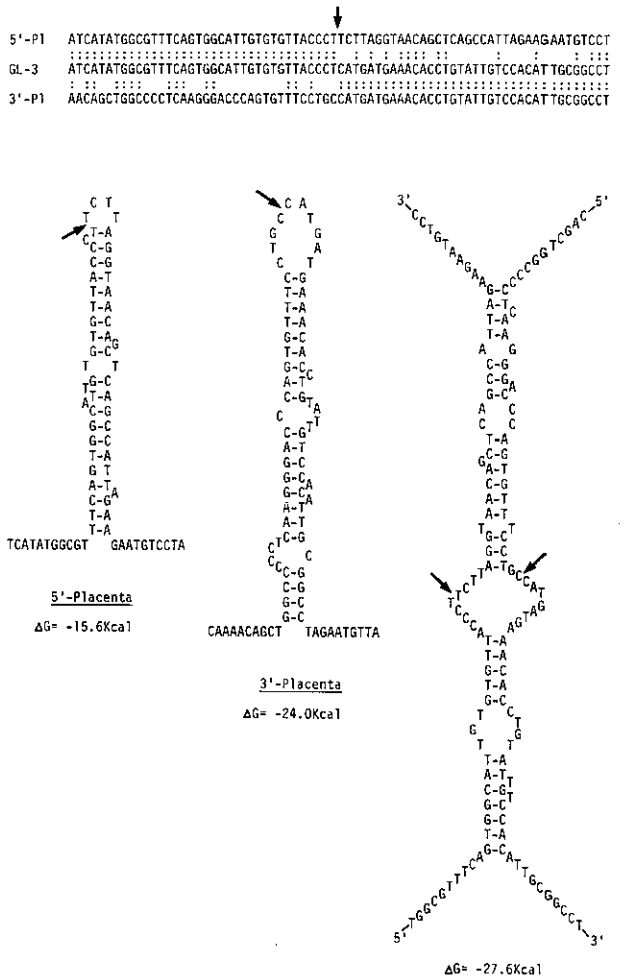


Fig. 2. Restriction map of the rearranged DNAs in EGF receptor gene in GL-3 and GL-5. In a Southern blotting (upper panel) where the cellular DNA was digested with *Hin*dIII and probed with 0.7 kb EGF receptor cDNA corresponding to the middle portion of the ligand binding domain, GL-3 gave a rearranged and amplified band of 24 kb, GL-5 gave a rearranged and amplified band of 15 kb, and placenta showed a normal nonamplified band of 17 kb. The solid box indicates the 7th exon (117 bp long, nucleotide residues 1076 to 1192 in the published EGF receptor sequence[16]). The restriction sites are abbreviated as follows: E, *Eco*RI; H, *Hind*III; B, *Bam*HI; S, *Sac*I; P, *Pst*I; A, *Ava*I.



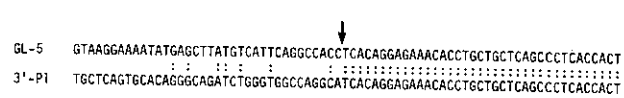


Fig. 3. Nucleotide sequence analysis of the deletion mutation in EGF receptor gene in glioblastoma multiforme. a) and c) Nucleotide sequences of the recombination junction in GL-3 and GL-5 EGF receptor genes. Closed triangles indicate breakpoints. b) Hypothetical secondary structures of the nucleotide sequences at the rearrangement in the GL-3 EGF receptor gene. The overall free energy (ΔG) was calculated as described.[21]

region from the amino terminus to the carboxyl terminus was isolated and sequenced by the dideoxy-nucleotide method.[13]

The primary structure of GL-5 EGF receptor cDNA was compared with that reported by Ullrich *et al.*[16] In accordance with our previous results, which suggest a deletion mutation(s) in the molecule,[7] GL-5 cDNA carried a large deletion within the extracellular domain of 801 bp [nucleotide residues 275 to 1075 in the published EGF receptor sequence[16]] (Fig. 1). The start site for this deletion in the cDNA molecule was essentially the same as the 3′-end of the 1st exon of EGF receptor gene in the genomic DNA.[17] Furthermore, as described later, the terminal site for this deletion in the cDNA was the 5′-terminus of the 7th exon of EGF receptor gene, based on the nucleotide sequencing analysis of placenta genomic DNA and on the physical map of human EGF receptor gene reported by Haley *et al.*[18] These results strongly suggest that the lack of the 801 bp stretch in the GL-5 EGF receptor cDNA is due to a deletion mutation running from a site within the 1st intron to a site in the 6th intron. The possibility of alternative splicing was ruled out by analysis of the deletion in the genomic DNA (see below).

In other regions of the receptor cDNA molecule including the transmembrane domain and the tyrosine kinase domain, no substitution of amino acids was found,

although two silent base changes were observed (Fig. 1). Thus, the predicted molecule of the altered EGF receptor in GL-5 still retains the original amino-terminal 29 amino acids including the entire signal peptide sequence of 24 amino acids, followed by a large deletion mutation in the ligand binding domain. Since the structurally altered EGF receptor in the membrane fraction of GL-5 cells showed a constitutive tyrosine kinase activity without its ligand, EGF, we conclude that the single deletion in the ligand binding domain is responsible for the functional activation of EGF receptor molecule in this human glioblastoma cell line.

Although we did not isolate the EGF receptor cDNA from GL-3, we suggest that a deletion mutation similar to that of GL-5 might also have occurred in GL-3 cDNA, based on the similarity of the size of mRNAs, products and the levels of kinase activity between GL-3 and GL-5.[7] A similar activation mechanism of EGF receptor gene in animals, i.e. a deletion in the extracellular domain without other point mutations in the receptor molecule, has been reported in cases of chicken erythroblastosis induced by integration of avian retrovirus provirus DNA withih the middle portion of the EGF receptor gene.[10]

Characterization of deletion mutation of EGF receptor gene in the genomic DNA To examine the nucleotide sequences involved in the formation of a deletion mutation in the genomic DNA, rearranged DNA fragments of the EGF receptor gene in GL-3 and GL-5 glioblastomas and the corresponding regions in human placenta genomic DNA were molecularly cloned (Fig. 2). Comparison of the sequences between placenta and glioblastomas demonstrated that the recombination point in GL-3 was present 370 bp upstream from the 7th exon, while that in GL-5 was 1.2 kb upstream from the same exon. Since the 6th exon of EGF receptor gene is located further upstream,[18] the recombination sites of EGF receptor gene in these two glioblastomas are present within the same intron, but at different positions.

Using the sequence just upstream from the recombination point in GL-3-rearranged DNA fragment as a probe (Fig. 3b), the 5′-donor sequence for recombination was molecularly cloned from a human genomic DNA library. Since the 1st intron in the human EGF receptor gene is quite large, we have not yet precisely located the 5′-donor sequences within this 1st intron region. However, our preliminary results suggest that the recombination point in the 1st intron is located more than 10 kb downstream from the 1st exon (Yamazaki and Shibuya, unpublished). The donor and the acceptor sequences for generation of GL-3 recombination and the acceptor sequence for GL-5 recombination are shown in Fig. 3a and c. Since only a limited nucleotide homology was observed between donor and acceptor regions, a homologous re-

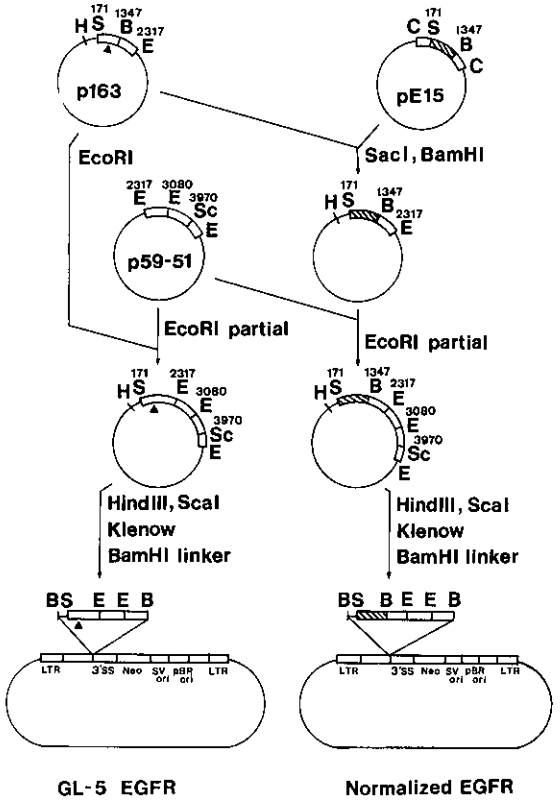


Fig. 4. Construction of the expression plasmids carrying the mutated EGF receptor cDNA. *Sac*I-*Bam*HI fragment of GL-5 EGF receptor cDNA in plasmid p163, which includes the whole deletion mutation from 275 to 1075, was replaced by *Sac*I-*Bam*HI fragment of normal EGF receptor cDNA derived from plasmid pE15.[17] Partially digested *Eco*RI fragments of p59-51 were ligated to these plasmids. *Hin*dIII-*Sca*I fragments of the plasmids which contain the coding regions were introduced into the *Bam*HI site of the expression vector pZIPneo-SV(X)I.[14] The restriction sites are abbreviated as follows: E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; S, *Sac*I; C, *Cla*I; Sc, *Sca*I.

Table I. Focus Formation of NIH/3T3 Cells with EGF Receptor cDNAs

Plasmid	No. of transformed foci per plate		
	−EGF	+EGF	+TGFα
pZIP	0, 1	1, 1	0, 1
pZIP-ER[a)]	0, 0	20, 21	17, 30
pZIP-ERM[b)]	0, 1	0, 1	0, 0

a) pZIPneo plasmid containing normal human EGF receptor cDNA.
b) pZIPneo plasmid containing GL-5 EGF receptor cDNA. EGF, 20 ng/ml; TGFα, 10 ng/ml; plasmid DNA, 5 μg/plate. Each experiment was done with 2 plates.

combination mechanism seemed unlikely in GL-3. Further, no octamer-nonamer-like sequences were detected near the recombination points in either GL-3 or GL-5, though such sequences are known to have important roles in the rearrangement of immunoglobulin gene and in the recombination between c-*myc* and immuno globulin heavy chain genes in Burkitt lymphoma.[19]

On the other hand, the regions including the recombination sites in GL-3 could form stem-loop structures as shown in Fig. 3b. The free energies of these stem loops at the donor and the acceptor regions for deletion mutation were −15.6 kcal and −24.0 kcal, respectively. In addition, a base-pairing between the donor and acceptor regions is also possible because of some nucleotide homology between these two regions (Fig. 3b, right). Although the decrease of free energy in this pairing is slightly larger than those in the stem loops, the physiological significance is still not clear since these two regions are located more than 10 kb from each other in the genomic DNA.[18] These results might indicate that a

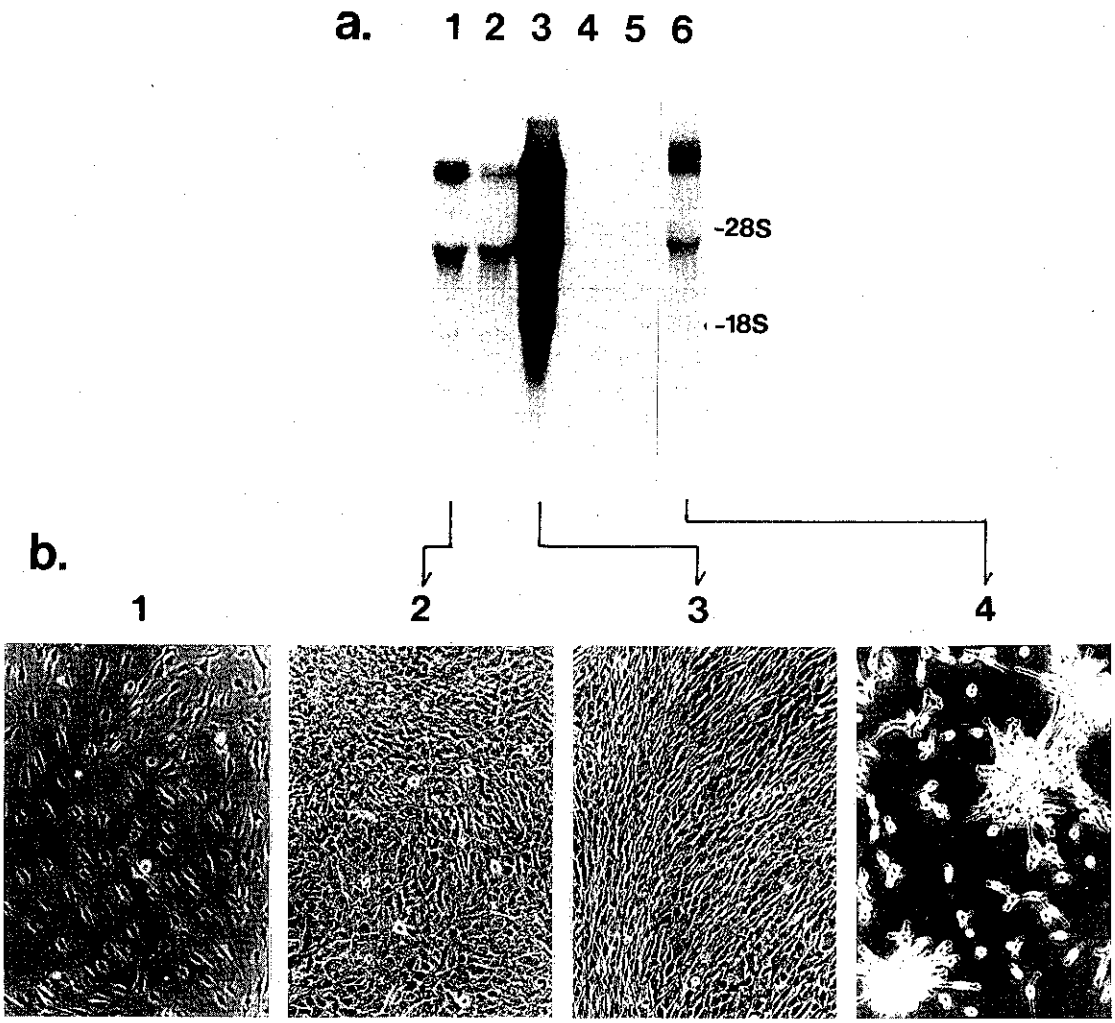


Fig. 5. Transforming activity of the mutated EGF receptor cDNA obtained from GL-5. a) Expression of EGF receptor gene in NIH-3T3 cells transfected with mutated or normal human EGF receptor cDNA. Lanes 1–5; single cell-derived NIH/3T3 cells transfected with mutated GL-5 EGF receptor cDNA (lane 1, clone #5-2; lane 3, clone #5-1). Lane 6; NIH/3T3 cell clone overexpressing the exogenously introduced normal EGF receptor. b) Morphology of the NIH/3T3 cells overexpressing the mutated or normal EGF receptor. Control NIH3T3 cells in Dulbecco's modified essential medium (DMEM) containing 6% newborn calf serum (1), #5-2 (2) and #5-1 (3) clones in DMEM, and the normal EGF receptor overexpressing NIH3T3 clone in the presence of 20 ng/ml EGF (4). The morphology of the #5-1 and #5-2 cells was not changed in the presence or absence of EGF.

unique recombination mechanism is involved in the activation process of EGF receptor gene in human brain tumors.

Transforming activity of deletion-carrying EGF receptor cDNA obtained from a glioblastoma multiforme To examine the transforming activity of the structurally altered GL-5 EGF receptor cDNA, this cDNA molecule was introduced into pZIPneo retrovirus vector[14] (Fig. 4) and transfected to NIH/3T3 fibroblast cells. In a regular focus-formation assay, no clear transformed foci were detected with the GL-5 cDNA-pZIP, although the full-length normal EGF receptor cDNA in the same vector exhibited a clear transforming activity in the presence of a high concentration of EGF (Table I, Fig. 5).

To detect a weak transforming activity, NIH/3T3 cells transfected with GL-5 cDNA-pZIP were screened with G418 resistance marker, and the resistant cells were examined in terms of the morphological alteration and the expression of the exogenously introduced GL-5 EGF receptor mRNA. Among 20 colonies tested, one colony was found to express a significant amount of GL-5 mRNA. From this colony, 5 clones each derived from a single cell were established and the levels of the GL-5 EGF receptor mRNA in these cells were further examined. As shown in Fig. 5, clone #5-1 expressed a high level of GL-5 EGF receptor mRNA and #5-2 expressed a moderate level. These two clones especially #5-1 clone, showed morphological transformation, although the degree was lower than that of normal EGF receptor-overexpressing NIH/3T3 cells in the presence of a high concentration of EGF (Fig. 5). As a control, normal EGF receptor-overexpressing cells without the ligand EGF did not show any morphological transformaton under these culture conditions.

As expected, an EGF-independent elevation of tyrosine kinase activity was confirmed in the GL-5 EGF receptor molecule expressed in clone #5-1 cells (Hino and Shibuya, unpublished). From these results, we consider that a weak but significant transforming activity is associated with the mutated GL-5 EGF receptor gene carrying a large single deletion within the ligand binding domain. This incomplete transforming activity is not surprising: the size of the truncation in the EGF receptor molecule appears to correlate with the transforming ability of overexpressed EGF receptor.[20] Thus, a structural alteration of EGF receptor gene observed in a population of human glioblastoma multiforme might have an important role in the process of malignant transformation of glial cells in humans.

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