EGFR Intron Recombination in Human Gliomas: Inappropriate Diversion of V(D)J Recombination?

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Abstract: The epidermal growth factor receptor (EGFR) is a membrane-bound, 170 kDa, protein tyrosine kinase that plays an important role in tumorigenesis. The EGFR gene, which is composed of over 168 kb of sequence, including a 123-kb first intron, is frequently amplified and rearranged in malignant gliomas leading to the expression of oncogenic deletion (DM) and tandem duplication (TDM) mutants. The most common DM in gliomas is EGFRvIII, which arises from recombination between introns 1 and 7 with deletion of exons 2 through 7 and intervening introns. In addition, some human gliomas express 180- to 190-kDa TDM, which are constitutively active and highly oncogenic. Both DM and TDM arise by recombination of introns that contain sequences with homology to the recombination signal sequence (RSS) heptamers and nonamers present in the V(D)J region of the immunoglobulin and T lymphocyte antigen receptor genes. V(D)J RSS have also been identified in certain proto-oncogenes like bcl-2 that are involved in translocations associated with the development of human lymphomas and in other genes such as hypoxanthine-guanine phosphoribosyl transferase (HPRT) in which deletion mutations and intron rearrangements are a common phenomenon. Together with the expression of recombination associated gene (RAG) and nonhomologous end-joining (NHEJ) proteins in gliomas, these observations suggest that aberrant activity of the V(D)J recombinase may be involved in the activation of proto-oncogenes in both liquid and solid tumors.

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I. THE EGFR GENE

The human EGFR locus spans 186 kb on chromosome 7. The gene encodes four extracellular cysteine-rich domains, two of which are involved in ligand binding. The remainder of the gene encodes a transmembrane (TM) domain, a protein tyrosine kinase (TK), a region that mediates calcium-induced receptor internalization (CAIN), and a regulatory (REG) domain with internal tyrosine phosphorylation sites. Analysis of the avian EGFR gene sequence strongly suggests that a portion of the extracellular region underwent tandem duplication at some point during phylogenetic evolution [1]. Similarly, the *Drosophila* EGFR gene contains three copies of the cysteine rich extracellular domain [2]. Thus, receptor diversification during phylogenetic evolution appears to have been mediated in part by tandem duplication of specific portions of the molecule.

II. EGFR AND MALIGNANT GLIOMAS

Malignant gliomas have many genetic alterations, including abnormalities of EGFR gene structure and copy number. These include amplification and rearrangement of the EGFR gene and high-level EGFR expression. A number of EGFR point mutations have been described in solid tumors, many of which lead to constitutive activation of the receptor [3]. Frequently, high-grade gliomas have EGFR gene amplification in the presence of other specific genetic alterations,

including inactivation of p53, PTEN, p16INK4a and LOH 10q [4-5]. Amplification of the EGFR gene occurs more frequently in the gliomas of older patients and is associated with a poor prognosis [3,6-7]. Although seen occasionally in other malignant gliomas, such as anaplastic astrocytoma, EGFR amplification tends to be less extensive than in glioblastoma multiforme [3]. However, it is not clear whether alterations in EGFR gene structure or expression provide a prognostic variable that is independent of age [7].

III. ONCOGENIC EGFR DELETION MUTATIONS

Amplification of the EGFR gene occurs in at least 40% of malignant gliomas [8-13]. Transcription of multiple gene copies per cell is thought to produce high-level EGFR expression in gliomas in comparison to normal glial cells [10]. Ectodomains of the other HER-family proteins can act as dominant negative regulators to inactivate heterodimers formed with EGFR, leading to inhibition of anchorage-independent growth of glioma cells [14]. Differential splicing of the EGFR transcript in rat liver produces a secreted 100-kDa form of EGFR that corresponds to the extracellular portion of the molecule [15,16]. Expression of this soluble EGFR molecule by normal tissues is inhibited by tumor-specific EGFR expression and is inversely related to tumor burden. Thus, the 110-kDa human form of the molecule may serve as a useful clinical marker for tumor recurrence in patients with ovarian carcinoma [17]. The role of this marker in glioma patients has yet to be assessed.

EGFR and its principle ligands, (EGF and TGF α) are expressed in a temporal and spatial pattern during development of the brain [16,17-19]. Expression of either ligand can

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lead to autocrine-stimulated cellular proliferation. When in the context of other genetic alterations, activation of the receptor may stimulate tumor cell growth [20-25]. These observations appear to have important implications for the role of stimulated wild type EGFR and constitutively active mutant EGFR forms in the promotion of malignant gliomas and other tumors in humans [23,26-30].

Although a number of oncogenic point mutations occur in the EGFR gene of malignant gliomas, the constitutively active deletion mutation known as EGFRvIII appears to be the most common activating mutation found to date. Other EGFR deletion mutations (DM) have been described which arise from specific gene rearrangements and internal deletions [10,11,13,31-33]. Most deletion mutations involve the loss of specific groups of exons encoding parts of the extracellular portion, and in some cases the intracellular portion, of the EGFR molecule [32,34].

A number of the glioma-derived EGFR deletion mutants are well characterized. An extensive N-terminal truncation of EGFR, commonly known as EGFRvI, has been reported in a small number of human gliomas (Fig. 1). This mutation creates a molecule that is quite similar to the v-erbB1 oncogene product, which induces malignant transformation *via* potent

corresponding to amino acids 6 through 273. This gene produces a 140- to 145-kDa receptor with unique epitopes. It is of substantial interest due to its effects on signal transduction and as a potential tumor-specific target [25,33,35-39]. Although EGFRvIII cannot bind ligand with high affinity, it is constitutively autophosphorylated [40]. EGFRvIII promotes glioma cell invasion which is downregulated by transfection of a phosphatase-active form of PTEN but not by inactive PTEN. Consequently, PTEN inhibits glioma cell invasion, even in the presence of the constitutively active EGFRvIII [41].

IV. EGFRvIII AND INTRONS 1 AND 7

EGFRvIII is the most common deletion mutant found in human gliomas. Much attention has been devoted to the functional activity of this particular mutant, which is constitutively active and oncogenic. Prior to complete sequencing of the EGFR locus, EGFRvIII was thought to arise from differential splicing of the wild type transcript. The Human Genome Project provided the sequence of EGFR intron 1, which is 123 kb in size (Fig. 2). Subsequently, Frederick *et al.* demonstrated that at least some human gliomas with EGFRvIII expression contain a rearranged EGFR gene with recombination between introns 1 and 7 [42]. Sequence analysis of introns 1 and 7 reveals elements from the 5'-end of intron 1 linked to a portion of the 3'-end of intron 7 (Fig. 3). Frederick *et al.* hypothesized that recombination might be related to the presence of highly repetitive Alu sequences in introns 1 and 7, a mechanism that has been implicated in gene inactivation in tumors previously [42].

In addition to recombination in tumors, intron 1 is probably involved in transcriptional regulation of EGFR expression under normal physiologic states. DNase hypersensitivity regions have been detected in this intron, which may indicate the presence of enhancer elements. With transcriptional upregulation of the EGFR gene, the DNase hypersensitivity regions are lost, further suggesting the presence of a transcriptional repressor [43]. The details of these regulatory elements in intron 1 have not yet been defined.

V. EGFR TANDEM DUPLICATION MUTANTS

In addition to wild type EGFR and the various deletion mutants found in human gliomas, a number of other EGFR-reactive species have been reported, which are actually larger than the wild type molecule [44,45]. We have characterized a number of these EGFR species in human glioma cell lines and in gliomas removed at surgery [46-48]. Many human glioma cell lines express the 170 kDa wild type EGFR. In addition, A172 human glioma cells express a large (190 kDa) EGFR mutant. This mutant receptor is encoded by a 11.5-kb, mRNA transcript which is expressed together with the wild type 10.5 kb EGFR transcript [46]. This mutation contains an internal tandem duplication of exons 18 through 26 which encode the tyrosine kinase (TK) and calcium-mediated internalization (CAIN) domains of the molecule [46-48]. Tumor cells that express this TDM also have an EGFR gene with an intron derived from a recombination event involving introns 26 and 17 [46]. Expression of this particular EGFR mutant in NR6 fibroblasts produces ligand-independent colony formation in soft agar and rapid tumor

Fig. (1). Major glioma-derived EGFR DM and TDM that have been characterized to date. Duplicated and deleted protein structural domains are indicated in comparison to the wild type EGFR.

constitutive receptor activation [13]. A mutation known as EGFRvII contains an in-frame deletion of 83 amino acids contained within domain IV of the molecule encoded by exons 14 and 15 (amino acids 520-603). EGFRvII remains capable of binding ligand and has enhanced tyrosine kinase activity [32]. A third mutation (EGFRvIII) is the most common deletion mutant expressed in malignant gliomas and has been reported to occur in almost half of such tumors [32,33]. EGFRvIII contains an in-frame deletion of exons 2 through 7

Fig. (2). Genomic structure of the human EGFR gene with exons and introns related to protein structural domains. Numbers indicate exons and arrows point to sites of intron recombination producing the major DM and TDM of EGFR. Introns containing CAC(A/T)GTG heptamers are shaded in black.

Fig. (3). Genomic PCR strategy used with human glioma genomic DNA to analyze intron recombination. **(A)** Ethidium-stained gel of chimeric intron from glioblastoma expressing EGFRvIII protein (lane 1). **(B)** Proposed model for recombination between intron 1 and 7 in EGFRvIII genes.

growth in nude mice [47]. The 190 kDa tandem duplication mutant (EGFR.TDM/18-26) is poorly downregulated by ligand and constitutively autophosphorylated. Together these factors account for its potent oncogenicity [47].

A second type of TK/CAIN tandem duplication mutant is also present in some glioma cell lines. In particular, KE and A1235 glioma cell lines express a large EGFR transcript along with that of the wild type EGFR molecule. The mutant transcripts in these cell lines carry a similar but distinct duplication of exons 18 through 25. This TDM contains a duplication of the tyrosine kinase domain and a portion of the CAIN domain. It is derived from a mutant EGFR gene with recombination between introns 25 and 17 [46,47]. As with EGFR.TDM/18-26, EGFR.TDM/18-25 exhibits impaired downregulation and constitutive autophosphorylation resulting in potent ligand-independent oncogenicity.

EGFR tandem duplication mutations also occur within the extracellular region of the molecule [48]. We identified a 180-kDa TDM in a human glioblastoma together with co-expression of EGFRvIII. This large mutant is designated EGFR/TDM.2-7 because it is encoded by a transcript containing a tandem duplication of exons 2 through 7. Like the TK/CAIN tandem duplication mutants, EGFR/TDM.2-7 maintains high basal phosphorylation in the absence of ligand. Despite significant alteration in the extracellular region of the molecule, EGFR/TDM.2-7 remains capable of binding ligand leading to basal phosphorylation that is induced further by ligand. Thus, together the three EGFR TDM identified to date represent a distinct class of EGFR mutants with strong oncogenic potential due to constitutive receptor activation. Each is associated with the presence of mutant introns that indicate a recombination event with subsequent rearrangement of the gene that unequivocally identifies

its origin. Recently, multiple examples of EGFR.TDM/18-25 and EGFR.TDM/18-26 have been recovered from human glioma specimens [34,49]. In addition, a novel EGFR.TDM/17-E8 consisting of a duplicated intron 7 through exon 8 resulting in an EGFR transcript truncated at amino acid 315 has been detected in a human glioma [49].

Like the tandem duplication mutants and EGFRvIII, the other EGFR deletion mutants probably arise from recombination between introns with deletion of intervening sequences. Moreover, the simultaneous expression of both deletion mutant (EGFRvIII) and tandem duplication mutant (EGFR.TDM/2-7 and EGFR.TDM/18-26) in human gliomas suggests a close relationship between the mechanisms underlying deletion and tandem duplication mutations. Although rare, the occurrence of tumors with genes that contain both deletion and tandem duplication of the same set of exons (i.e. EGFRvIII and EGFR.TDM/2-7) further supports this view.

The EGFR gene is prone to oncogenic activation by way of many different structural alterations. Activating mutations other than deletion and tandem duplication have also been reported, including a number of different C-terminal truncations affecting function of the regulatory domain. One such mutation involves the loss of 255 bases of the C-terminal tail [50]. Other C-terminal deletions have been noted as well [51]. In addition, small internal deletions and a variety of point mutations within the tyrosine kinase domain have been reported [52,53].

VI. EGFR INTRON RECOMBINATION AND V(D)J RECOMBINATION SIGNAL SEQUENCES (RSS)

The DM and TDM identified to date arise from recombination between introns that flank the deleted and duplicated regions of the EGFR gene. The introns from which the TDM and DM arise contain a number of conserved DNA sequence elements (Fig. 4). Each intron contains two perfectly conserved heptamers, one with the sequence CACAGTG and one with CACTGTG. Although the breakpoint in each intron varies from one tumor to another, in the case of the tandem duplication mutants, both heptamers are found on the 5' side of the recombination site. These heptameric sequences correspond to the recombination signal sequences present in the V(D)J region of the immunoglobulin and T cell receptor genes. V(D)J recombination is known to require these sequences. In addition, classical V(D)J recombination involves a conserved nonameric sequence ACAAAAACC which is also found in many EGFR introns that undergo recombination. To date, EGFR introns that do not contain V(D)J heptamers and nonamers have not been reported to give rise to DM and TDM (Fig. 4).

Conserved V(D)J heptamers appear repeatedly within certain EGFR introns (Fig. 4). It is possible that the number of heptamers present in each intron might be related to the relative frequency of the different mutations. The presence of 38 perfectly conserved heptamer copies and 3 perfect nonamers in intron 1, and the high incidence of the EGFRvIII mutation involving introns 1 and 7 in gliomas, lend circumstantial evidence to this possibility.

Introns 1 and 7, which recombine to form EGFRvIII genes, contain copies of Alu repetitive sequences [42]. These

repetitive elements are known to be associated with recombination events in a number of other genes. In contrast to the frequent occurrence of V(D)J heptamers and nonamers in EGFR introns involved in tandem duplication, we could find no examples of Alu or LINE sequences in introns 17, 25 or 26 [21,54]. Therefore, it seems unlikely that such sequences and their associated recombination processes could give rise to TDM. The answer is less clear for the EGFR deletion mutants, however.

The most notable examples of somatic cellular genes that undergo recombination are the T cell antigen receptor and immunoglobulin genes in which a domain known as the V(D)J or variable-diversity-joining region mediates this recombination. V(D)J recombination occurs in lymphocytes as part of the activation and diversification of the immunological response to foreign antigens [55-58]. A number of protein factors are important components of this process, including the recombination associated gene or RAG proteins [55-67]. RAG1 and RAG2 mediate recombination of V(D)J sites through recognition of recombination signal sequences within introns adjacent to the actual site of cleavage [60,65]. These proteins act through heptamer sequences CAC(A/T)GTG in association with the nonamer sequence ACAAAAACC, which is also present in close proximity to V(D)J recombination sites [65]. The efficiency of the process of V(D)J recombination is affected by the presence of additional proteins, including some that are important in DNA repair [65,68,69-72]. Recombination at the V(D)J locus also requires a precise spatial relationship between RSS motifs.

VII. RAG AND NHEJ PROTEIN MACHINERY EXPRESSION IN GLIOMAS

Although RAG proteins are primarily expressed in activated lymphocytes, they have also been found in the developing nervous system, where their exact function remains unknown [73-75]. RAG2 has also been observed in murine brain tumors [76]. The expression of these proteins in the developing nervous system and in brain tumors, in particular, is of interest with respect to data on recombination between EGFR introns and the presence of V(D)J heptamer and nonamer sequences found in those introns (Fig. 4). Thus, the detection of RAG proteins in the central nervous system raises questions about their possible function in the brain and whether recombination might occur "aberrantly" in genes not specifically involved in immunological diversification *via* RAG-mediated mechanisms.

The two major pathways for repairing double-strand DNA breaks (DSB), homologous recombination and non-homologous end joining (NHEJ), have traditionally been thought to operate in different stages of the cell cycle. DSB created during V(D)J recombination rely on classical NHEJ to repair broken ends [61-67]. However, mutations that form unstable RAG post-cleavage complexes allow DNA ends to participate in both homologous recombination and the error-prone alternative NHEJ pathway [61-67]. RAG-mediated nicks efficiently stimulate homologous recombination and promote oncogenic chromosomal rearrangements. DSB breaks are created in lymphocytes to generate the antigen receptor and antibody repertoire of lymphocytes [61-67]. After DSB are created, the DNA joining required to com-

Fig. (4). EGFR introns involved in the major DM and TDM. V(D)J heptamer sequence positions are indicated by arrows: CACAGTG, CACTGTG, and CACATGTG. Intron breakpoints are indicated by black arrowheads below each intron

plete the process is carried out by the NHEJ pathway [61-67]. The NHEJ machinery is present not only in lymphocytes, but in all eukaryotic cells. The NHEJ pathway is needed to repair physiologic breaks, as well as the pathologic breaks that arise from different DNA damaging processes [61-67]. RAG and NHEJ proteins are also expressed in malignant glioma cells (our unpublished observations) suggesting that the components necessary for mediating NHEJ are available for repair of RAG-mediated DSB created in malignant glioma cells.

VIII. HPRT GENE DELETIONS AND V(D)J RECOMBINASE

Major deletions in such genes as that of hypoxanthine-guanine phosphoribosyl transferase (HPRT) appear to arise in association with V(D)J RSS-mediated activity. Somatic mutations in the HPRT housekeeping gene rarely occur in the lymphocytes of normal individuals. In children less than 5 years of age, 30-40% of these genomic HPRT mutations have a 20-kb deletion of exons 2 and 3 [77]. Fuscoe *et al.* sequenced the breakpoint sites for these specific deletions in lymphocyte clones isolated from 13 normal newborns and identified 3 classes of deletions. Each class had the same intron 1 breakpoint, but different breakpoints in intron 3. Sequence analysis of the HPRT DM strongly suggest the possibility of a V(D)J-like recombination event. Certain sequences in the HPRT deletion joining regions are analogous to the N-nucleotide insertion hypervariable regions of rearranged T-cell receptor (TCR) genes. Many of these malignancies, including acute (T cell) lymphocytic leukemia (ALL), are also characterized by V(D)J recombinase-mediated recombination in critical regions of the genome.

The HPRT gene appears to be the object of a mutagenic mechanism leading to harmless mutations that may be tumorigenic when they occur in other genes [78]. The authors of these studies have suggested that unregulated expression of V(D)J recombinase activity may be an important mechanism for genomic rearrangement in the genesis of cancer. HPRT mutations are also found in patients with Fanconi's anemia (FA), a disorder characterized by chromosomal breakage and a high incidence of deletion mutations. The principal cellular feature of Fanconi anemia, an inherited cancer-prone disorder, is frequent chromosomal breakage. HPRT deletions observed in FA patients appear to arise from poor fidelity of V(D)J coding joint formation and inaccurate repair of DSB by NHEJ processes [79].

IX. CHROMOSOMAL TRANSLOCATION IN LEUKEMIA AND LYMPHOMA

Genomic instability is a universal characteristic of malignancy. Human hematopoietic malignancies are frequently characterized by chromosomal translocations in which the T cell antigen receptor or immunoglobulin genes are brought into the proximity of certain proto-oncogenes. As a result, specific gene rearrangements may be associated with the development of particular tumor types [80]. One example of this phenomenon is Burkitt's lymphoma in which translocation t(8;14) results from aberrant V(D)J recombination which brings the IgH transcriptional enhancer into close proximity with the *c-myc* gene. Similarly, mice that are deficient in both a nonhomologous end joining (NHEJ) DNA repair protein and p53 develop lymphomas with an IgH/*c-myc* fusion. In this animal model, RAG-mediated breaks during V(D)J recombination lead to break-induced-replica-

tion with resulting juxtaposition of the IgH and c-myc loci [81].

The translocation t(14;18) that is present in 90% of follicular lymphomas produces aberrant activation of the BCL-2 gene [82]. This translocation arises from recombination between the BCL-2 gene and the immunoglobulin heavy chain (IgH) locus. Due to the presence of the D(H) and J(H) gene segments from the IgH locus, this particular translocation is thought to result from a mistake occurring during V(D)J recombination in B lymphocytes.

The t(11;18) translocation, which fuses the API2 and MALT1 genes, is one of the most frequent chromosomal translocations associated with mucosa-associated lymphoid tissue (MALT) lymphomas. The breakpoints present in this translocation have been cloned and characterized [83]. The API2 breakpoint has been identified within intron 7 and found to possess heptamers of immunoglobulin V(D)J recombination signal sequences (RSS) suggesting that the t(11;18) translocation in MALT lymphomas might be mediated by aberrant V(D)J recombination. Thus, there are a growing number of examples of apparent inappropriate diversion of V(D)J recombination machinery accounting for DNA translocations in lymphoid malignancies which may constitute a source of oncogenic activation [62]. Although V(D)J-type recombination has been implicated in genetic translocation leading to lymphoid malignancies, it has not yet been proven conclusively to be a mechanism by which growth factor receptor genes are oncogenically activated in solid tumors.

CONCLUSION

In the last decade, accumulating evidence has suggested that infidelity of the V(D)J recombinase and NHEJ pathways may account for genomic alterations in a number of different disease states. Genomic sequence data indicate a strong association between the presence of V(D)J heptamer and nonamer motif distribution and the occurrence of recombination between EGFR introns in malignant gliomas. This association suggests that V(D)J recombination signal sequences could mediate the formation of oncogenic EGFR deletion and tandem duplication mutants in human gliomas. These observations and the observed expression of RAG and NHEJ proteins in malignant gliomas suggest that aberrant activity of the V(D)J recombinase may be specifically involved in oncogenic activation of the EGFR gene in human malignant gliomas.

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REFERENCES

- Callaghan, T., Antczak, M., Flickinger, T., Raines, M., Myers, M., Kung, H. J. A complete description of the EGF-receptor exon structure: implication in oncogenic activation and domain evolution. *Oncogene* **1993**, *8*: 2939-2948.
- Price, J. V., Clifford, R. J., Schupbach, T. The maternal ventralizing locus torpedo is allelic to faint little ball, an embryonic lethal, and encodes the Drosophila EGF receptor homolog. *Cell* **1989**, *56*: 1085-1092.
- Hurt, M. R., Moosy, J., Donovan-Peluso, M., Locker, J. Amplification of epidermal growth factor receptor gene in gliomas: histopathology and prognosis. *J. Neuropathol. Exp. Neurol.* **1992**, *51*: 84-90.
- Rich, J. N., Hans, C., Jones, B., Iversen, E. S., McLendon, R. E., Rasheed, B. K., Dobra, A., Dressman, H. K., Bigner, D. D., Nevins, J. R., West, M. Gene expression profiling and genetic markers in glioblastoma survival. *Cancer Res.* **2005**, *65*: 4051-4058.
- Homma, T., Fukushima, T., Vaccarella, S., Yonekawa, Y., Di Patre, P. L., Franceschi, S., Ohgaki, H. Correlation among pathology, genotype, and patient outcomes in glioblastoma. *J. Neuropathol. Exp. Neurol.* **2006**, *65*: 846-854.
- Frisa, P. S., Walter, E. I., Ling, L., Kung, H. J., Jacobberger, J. W. Stepwise transformation of astrocytes by simian virus 40 large T antigen and epidermal growth factor receptor overexpression. *Cell Growth Differ.* **1996**, *7*: 223-233.
- Diedrich, U., Lucius, J., Baron, E., Behnke, J., Pabst, B., Zoll, B. Distribution of epidermal growth factor receptor gene amplification in brain tumours and correlation to prognosis. *J. Neurol.* **1995**, *242*: 683-688.
- Bigner, S. H., Humphrey, P. A., Wong, A. J., Vogelstein, B., Mark, J., Friedman, H. S., Bigner, D. D. Characterization of the epidermal growth factor receptor in human glioma cell lines and xenografts. *Cancer Res.* **1990**, *50*: 8017-8022.
- Humphrey, P. A., Wong, A. J., Vogelstein, B., Friedman, H. S., Werner, M. H., Bigner, D. D., Bigner, S. H. Amplification and expression of the epidermal growth factor receptor gene in human glioma xenografts. *Cancer Res.* **1988**, *48*: 2231-2238.
- Liebermann, T. A., Nusbaum, H. R., Razon, N., Kris, R., Lax, I., Soreq, H., Whittle, N., Waterfield, M. D., Ullrich, A., Schlessinger, J. Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. *Nature* **1985**, *313*: 144-147.
- Malden, L. T., Novak, U., Kaye, A. H., Burgess, A. W. Selective amplification of the cytoplasmic domain of the epidermal growth factor receptor gene in glioblastoma multiforme. *Cancer Res.* **1988**, *48*: 2711-2714.
- Wong, A. J., Bigner, S. H., Bigner, D. D., Kinzler, K. W., Hamilton, S. R., Vogelstein, B. Increased expression of the epidermal growth factor receptor gene in malignant gliomas is invariably associated with gene amplification. *Proc. Natl. Acad. Sci. U. S. A.* **1987**, *84*: 6899-6903.
- Wong, A. J., Ruppert, J. M., Bigner, S. H., Grzeschik, C. H., Humphrey, P. A., Bigner, D. S., Vogelstein, B. Structural alterations of the epidermal growth factor receptor gene in human gliomas. *Proc. Natl. Acad. Sci. U. S. A.* **1992**, *89*: 2965-2969.
- O'Rourke, D. M., Qian, X., Zhang, H. T., Davis, J. G., Nute, E., Meinkoth, J., Greene, M. I. Trans receptor inhibition of human glioblastoma cells by erbB family ectodomains. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94*: 3250-3255.
- Basu, A., Raghunath, M., Bishayee, S., Das, M. Inhibition of tyrosine kinase activity of the epidermal growth factor (EGF) receptor by a truncated receptor form that binds to EGF: role for interreceptor interaction in kinase regulation. *Mol. Cell Biol.* **1989**, *9*: 671-677.
- Petch, L. A., Harris, J., Raymond, V. W., Blasband, A., Lee, D. C., Earp, H. S. A truncated, secreted form of the epidermal growth factor receptor is encoded by an alternatively spliced transcript in normal rat tissue. *Mol. Cell Biol.* **1990**, *10*: 2973-2982.
- Baron, A. T., Cora, E. M., Lafky, J. M., Boardman, C. H., Buenafe, M. C., Rademaker, A., Liu, D., Fishman, D. A., Podratz, K. C., Maihle, N. J. Soluble Epidermal Growth Factor Receptor (sEGFR/sErbB1) as a Potential Risk, Screening, and Diagnostic Serum Biomarker of Epithelial Ovarian Cancer. *Cancer Epidemiol. Biomarkers Prev.* **2003**, *12*: 103-113.
- Seroogy, K. B., Numan, S., Gall, C. M., Lee, D. C., Kornblum, H. I. Expression of EGF receptor mRNA in rat nigrostriatal system. *Neuroreport* **1994**, *6*: 105-108.
- Seroogy, K. B., Gall, C. M., Lee, D. C., Kornblum, H. I. Proliferative zones of postnatal rat brain express epidermal growth factor receptor mRNA. *Brain Res.* **1995**, *670*: 157-164.
- Blasband, A. J., Gilligan, D. M., Winchell, L. F., Wong, S. T., Luetteke, N. C., Rogers, K. T., Lee, D. C. Expression of the TGF alpha integral membrane precursor induces transformation of NRK cells. *Oncogene* **1990**, *5*: 1213-1221.

- [21] Ciesielski, M. J., Fenstermaker, R. A. Structure of the Epidermal Growth Factor Receptor Gene and Intron Recombination in Human Gliomas. *Curr. Genom.* **2003**, *4*: 1-12.
- [22] Di Marco, E., Pierce, J. H., Fleming, T. P., Kraus, M. H., Molloy, C. J., Aaronson, S. A., Di Fiore, P. P. Autocrine interaction between TGF alpha and the EGF-receptor: quantitative requirements for induction of the malignant phenotype. *Oncogene* **1989**, *4*: 831-838.
- [23] Chan, K. S., Carbajal, S., Kiguchi, K., Clifford, J., Sano, S., Di-Giovanni, J. Epidermal growth factor receptor-mediated activation of Stat3 during multistage skin carcinogenesis. *Cancer Res.* **2004**, *64*: 2382-2389.
- [24] Lee, D. C., Fenton, S. E., Berkowitz, E. A., Hissong, M. A. Transforming growth factor alpha: expression, regulation, and biological activities. *Pharmacol. Rev.* **1995**, *47*: 51-85.
- [25] Rodeck, U., Williams, N., Murthy, U., Herlyn, M. Monoclonal antibody 425 inhibits growth stimulation of carcinoma cells by exogenous EGF and tumor-derived EGF/TGF-alpha. *J. Cell Biochem.* **1990**, *44*: 69-79.
- [26] Maruno, M., Kovach, J. S., Kelly, P. J., Yanagihara, T. Transforming growth factor-alpha, epidermal growth factor receptor, and proliferating potential in benign and malignant gliomas. *J. Neurosurg.* **1991**, *75*: 97-102.
- [27] Holland, E. C., Hively, W. P., DePinho, R. A., Varmus, H. E. A constitutively active epidermal growth factor receptor cooperates with disruption of G1 cell-cycle arrest pathways to induce glioma-like lesions in mice. *Genes Dev.* **1998**, *12*: 3675-3685.
- [28] Louis, D. N., von Deimling, A., Chung, R. Y., Rubio, M. P., Whaley, J. M., Eibl, R. H., Ohgaki, H., Wiestler, O. D., Thor, A. D., Seizinger, B. R. Comparative study of p53 gene and protein alterations in human astrocytic tumors. *J. Neuropathol. Exp. Neurol.* **1993**, *52*: 31-38.
- [29] Lund-Johansen, M., Bjerkvig, R., Humphrey, P. A., Bigner, S. H., Bigner, D. D., Laerum, O. D. Effect of epidermal growth factor on glioma cell growth, migration, and invasion *in vitro*. *Cancer Res.* **1990**, *50*: 6039-6044.
- [30] Ueki, K., Ono, Y., Henson, J. W., Efird, J. T., von Deimling, A., Louis, D. N. CDKN2/p16 or RB alterations occur in the majority of glioblastomas and are inversely correlated. *Cancer Res.* **1996**, *56*: 150-153.
- [31] Batra, S. K., Castelino-Prabhu, S., Wikstrand, C. J., Zhu, X., Humphrey, P. A., Friedman, H. S., Bigner, D. D. Epidermal growth factor ligand-independent, unregulated, cell-transforming potential of a naturally occurring human mutant EGFRvIII gene. *Cell Growth Differ.* **1995**, *6*: 1251-1259.
- [32] Humphrey, P. A., Gangarosa, L. M., Wong, A. J., Archer, G. E., Lund-Johansen, M., Bjerkvig, R., Laerum, O. D., Friedman, H. S., Bigner, D. D. Deletion-mutant epidermal growth factor receptor in human gliomas: effects of type II mutation on receptor function. *Biochem. Biophys. Res. Commun.* **1991**, *178*: 1413-1420.
- [33] Wikstrand, C. J., McLendon, R. E., Friedman, A. H., Bigner, D. D. Cell surface localization and density of the tumor-associated variant of the epidermal growth factor receptor, EGFRvIII. *Cancer Res.* **1997**, *57*: 4130-4140.
- [34] Frederick, L., Wang, X. Y., Eley, G., James, C. D. Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas. *Cancer Res.* **2000**, *60*: 1383-1387.
- [35] Capala, J., Barth, R. F., Bailey, M. Q., Fenstermaker, R. A., Marek, M. J., Rhodes, B. A. Radiolabeling of epidermal growth factor with ^{99m}Tc and *in vivo* localization following intracerebral injection into normal and glioma-bearing rats. *Bioconjug. Chem.* **1997**, *8*: 289-295.
- [36] Capala, J., Barth, R. F., Bendayan, M., Lauzon, M., Adams, D. M., Soloway, A. H., Fenstermaker, R. A., Carlsson, J. Boronated epidermal growth factor as a potential targeting agent for boron neutron capture therapy of brain tumors. *Bioconjug. Chem.* **1996**, *7*: 7-15.
- [37] Fenstermaker, R. A., Capala, J., Barth, R. F., Hujer, A., Kung, H. J., Kaetzel, D. M., Jr. The effect of epidermal growth factor receptor (EGFR) expression on *in vivo* growth of rat C6 glioma cells. *Leukemia* **1995**, *9 Suppl 1*: S106-12.: S106-S112.
- [38] Humphrey, P. A., Wong, A. J., Vogelstein, B., Zalutsky, M. R., Fuller, G. N., Archer, G. E., Friedman, H. S., Kwatra, M. M., Bigner, S. H., Bigner, D. D. Anti-synthetic peptide antibody reacting at the fusion junction of deletion-mutant epidermal growth factor receptors in human glioblastoma. *Proc. Natl. Acad. Sci. U. S. A.* **1990**, *87*: 4207-4211.
- [39] Moscatello, D. K., Ramirez, G., Wong, A. J. A naturally occurring mutant human epidermal growth factor receptor as a target for peptide vaccine immunotherapy of tumors. *Cancer Res.* **1997**, *57*: 1419-1424.
- [40] Ekstrand, A. J., Longo, N., Hamid, M. L., Olson, J. J., Liu, L., Collins, V. P., James, C. D. Functional characterization of an EGF receptor with a truncated extracellular domain expressed in glioblastomas with EGFR gene amplification. *Oncogene* **1994**, *9*: 2313-2320.
- [41] Cai, X. M., Tao, B. B., Wang, L. Y., Liang, Y. L., Jin, J. W., Yang, Y., Hu, Y. L., Zha, X. L. Protein phosphatase activity of PTEN inhibited the invasion of glioma cells with epidermal growth factor receptor mutation type III expression. *Int. J. Cancer* **2005**, *117*: 905-912.
- [42] Frederick, L., Eley, G., Wang, X. Y., James, C. D. Analysis of genomic rearrangements associated with EGFRvIII expression suggests involvement of Alu repeat elements. *Neuro-oncol.* **2000**, *2*: 159-163.
- [43] Chrysogelos, S. A. Chromatin structure of the EGFR gene suggests a role for intron 1 sequences in its regulation in breast cancer cells. *Nucleic Acids Res.* **1993**, *21*: 5736-5741.
- [44] Panneerselvam, K., Kanakaraj, P., Raj, S., Das, M., Bishayee, S. Characterization of a novel epidermal-growth-factor-receptor-related 200-kDa tyrosine kinase in tumor cells. *Eur. J. Biochem.* **1995**, *230*: 951-957.
- [45] Steck, P. A., Lee, P., Hung, M. C., Yung, W. K. Expression of an altered epidermal growth factor receptor by human glioblastoma cells. *Cancer Res.* **1988**, *48*: 5433-5439.
- [46] Fenstermaker, R. A., Ciesielski, M. J., Castiglia, G. J. Tandem duplication of the epidermal growth factor receptor tyrosine kinase and calcium internalization domains in A-172 glioma cells. *Oncogene* **1998**, *16*: 3435-3443.
- [47] Ciesielski, M. J., Fenstermaker, R. A. Oncogenic epidermal growth factor receptor mutants with tandem duplication: gene structure and effects on receptor function. *Oncogene* **2000**, *19*: 810-820.
- [48] Fenstermaker, R. A., Ciesielski, M. J. Deletion and tandem duplication of exons 2 - 7 in the epidermal growth factor receptor gene of a human malignant glioma. *Oncogene* **2000**, *19*: 4542-4548.
- [49] Arjona, D., Bello, M. J., Rey, J. A. EGFR intragenic loss and gene amplification in astrocytic gliomas. *Cancer Genet. Cytogenet.* **2006**, *164*: 39-43.
- [50] Ekstrand, A. J., Sugawa, N., James, C. D., Collins, V. P. Amplified and rearranged epidermal growth factor receptor genes in human glioblastomas reveal deletions of sequences encoding portions of the N- and/or C-terminal tails. *Proc. Natl. Acad. Sci. U. S. A.* **1992**, *89*: 4309-4313.
- [51] Eley, G., Frederick, L., Wang, X. Y., Smith, D. I., James, C. D. 3' end structure and rearrangements of EGFR in glioblastomas. *Genes Chromo. Can* **1998**, *23*: 248-254.
- [52] Shimato, S., Mitsudomi, T., Kosaka, T., Yatabe, Y., Wakabayashi, T., Mizuno, M., Nakahara, N., Hatano, H., Natsume, A., Ishii, D., Yoshida, J. EGFR mutations in patients with brain metastases from lung cancer: association with the efficacy of gefitinib. *Neuro-oncol.* **2006**, *8*: 137-144.
- [53] Barber, T. D., Vogelstein, B., Kinzler, K. W., Velculescu, V. E. Somatic mutations of EGFR in colorectal cancers and glioblastomas. *N. Engl. J. Med.* **2004**, *351*: 2883.
- [54] Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., Lipman, D. J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **1997**, *25*: 3389-3402.
- [55] Bailin, T., Mo, X., Sadofsky, M. J. A RAG1 and RAG2 tetramer complex is active in cleavage in V(D)J recombination. *Mol. Cell Biol.* **1999**, *19*: 4664-4671.
- [56] McBlane, J. F., van Gent, D. C., Ramsden, D. A., Romeo, C., Cuomo, C. A., Gellert, M., Oettinger, M. A. Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps. *Cell* **1995**, *83*: 387-395.
- [57] Schatz, D. G., Oettinger, M. A., Baltimore, D. The V(D)J recombination activating gene, RAG-1. *Cell* **1989**, *59*: 1035-1048.
- [58] Silver, D. P., Spanopoulou, E., Mulligan, R. C., Baltimore, D. Dispensable sequence motifs in the RAG-1 and RAG-2 genes for plasmid V(D)J recombination. *Proc. Natl. Acad. Sci. U. S. A.* **1993**, *90*: 6100-6104.

- [59] Chen, C. L., Woo, M. H., Neale, G. A., Goorha, R. M., Fuscoe, J. C., Behm, F. G., Mathew, S., Relling, M. V. A human lymphoid leukemia cell line with a V(D)J recombinase-mediated deletion of hprt. *Mutat. Res.* **1998**, *403*: 113-125.
- [60] Difilippantonio, M. J., McMahan, C. J., Eastman, Q. M., Spanopoulou, E., Schatz, D. G. RAG1 mediates signal sequence recognition and recruitment of RAG2 in V(D)J recombination. *Cell* **1996**, *87*: 253-262.
- [61] Hiom, K., Gellert, M. Assembly of a 12/23 paired signal complex: a critical control point in V(D)J recombination. *Mol. Cell* **1998**, *1*: 1011-1019.
- [62] Hiom, K., Melek, M., Gellert, M. DNA transposition by the RAG1 and RAG2 proteins: a possible source of oncogenic translocations. *Cell* **1998**, *94*: 463-470.
- [63] Melek, M., Gellert, M., van Gent, D. C. Rejoining of DNA by the RAG1 and RAG2 proteins. *Science* **1998**, *280*: 301-303.
- [64] Notarangelo, L. D., Villa, A., Schwarz, K. RAG and RAG defects. *Curr. Opin. Immunol.* **1999**, *11*: 435-442.
- [65] Rodgers, K. K., Villey, I. J., Ptaszek, L., Corbett, E., Schatz, D. G., Coleman, J. E. A dimer of the lymphoid protein RAG1 recognizes the recombination signal sequence and the complex stably incorporates the high mobility group protein HMG2. *Nucleic Acids Res.* **1999**, *27*: 2938-2946.
- [66] Roth, D. B., Zhu, C., Gellert, M. Characterization of broken DNA molecules associated with V(D)J recombination. *Proc. Natl. Acad. Sci. U. S. A.* **1993**, *90*: 10788-10792.
- [67] Shockett, P. E., Schatz, D. G. DNA hairpin opening mediated by the RAG1 and RAG2 proteins. *Mol. Cell Biol.* **1999**, *19*: 4159-4166.
- [68] Nishikawa, R., Furnari, F. B., Lin, H., Arap, W., Berger, M. S., Cavenee, W. K., Su Huang, H. J. Loss of P16INK4 expression is frequent in high grade gliomas. *Cancer Res.* **1995**, *55*: 1941-1945.
- [69] Hernandez-Munain, C., Krangel, M. S. Regulation of the T-cell receptor delta enhancer by functional cooperation between c-Myb and core-binding factors. *Mol. Cell Biol.* **1994**, *14*: 473-483.
- [70] Hernandez-Munain, C., Lauzurica, P., Krangel, M. S. Regulation of T cell receptor delta gene rearrangement by c-Myb. *J. Exp. Med.* **1996**, *183*: 289-293.
- [71] Kawaichi, M., Oka, C., Shibayama, S., Koromilas, A. E., Matsunami, N., Hamaguchi, Y., Honjo, T. Genomic organization of mouse J kappa recombination signal binding protein (RBP-J kappa) gene. *J. Biol. Chem.* **1992**, *267*: 4016-4022.
- [72] Lauzurica, P., Zhong, X. P., Krangel, M. S., Roberts, J. L. Regulation of T cell receptor delta gene rearrangement by CBF/PEBP2. *J. Exp. Med.* **1997**, *185*: 1193-1201.
- [73] Chun, J. J., Schatz, D. G., Oettinger, M. A., Jaenisch, R., Baltimore, D. The recombination activating gene-1 (RAG-1) transcript is present in the murine central nervous system. *Cell* **1991**, *64*: 189-200.
- [74] Fuller, K., Storb, U. Identification and characterization of the murine Rag1 promoter. *Mol. Immunol.* **1997**, *34*: 939-954.
- [75] Weiner, J. A., Chun, J. Maternally derived immunoglobulin light chain is present in the fetal mammalian CNS. *J. Neurosci.* **1997**, *17*: 3148-3156.
- [76] Aoki, T., Tashiro, K., Miyatake, S., Nakano, T., Oda, Y., Kikuchi, H., Honjo, T. Expression of the RAG-2 gene in murine central nervous system tumor cell lines. *Biochem. Biophys. Res. Commun.* **1991**, *181*: 151-158.
- [77] Fuscoe, J. C., Zimmerman, L. J., Lippert, M. J., Nicklas, J. A., O'Neill, J. P., Albertini, R. J. V(D)J recombinase-like activity mediates hprt gene deletion in human fetal T-lymphocytes. *Cancer Res.* **1991**, *51*: 6001-6005.
- [78] Finette, B. A. Analysis of mutagenic V(D)J recombinase mediated mutations at the HPRT locus as an *in vivo* model for studying rearrangements with leukemogenic potential in children. *DNA Repair (Amst)* **2006**, *5*: 1049-1064.
- [79] Laquerbe, A., Sala-Trepat, M., Vives, C., Escarceller, M., Papadopoulos, D. Molecular spectra of HPRT deletion mutations in circulating T-lymphocytes in Fanconi anemia patients. *Mutat. Res.* **1999**, *431*: 341-350.
- [80] Le Beau, M. M., Rowley, J. D. Chromosomal abnormalities in leukemia and lymphoma: clinical and biological significance. *Adv. Hum. Genet.* **1986**, *15*: 1-54: 1-54.
- [81] Difilippantonio, M. J., Petersen, S., Chen, H. T., Johnson, R., Jasin, M., Kanaar, R., Ried, T., Nussenzweig, A. Evidence for replicative repair of DNA double-strand breaks leading to oncogenic translocation and gene amplification. *J. Exp. Med.* **2002**, *196*: 469-480.
- [82] Wyatt, R. T., Rudders, R. A., Zelenetz, A., Delellis, R. A., Krontiris, T. G. BCL2 oncogene translocation is mediated by a chi-like consensus. *J. Exp. Med.* **1992**, *175*: 1575-1588.
- [83] Sato, Y., Akiyama, Y., Tanizawa, T., Shibata, T., Saito, K., Mori, S., Kamiyama, R., Yuasa, Y. Molecular characterization of the genomic breakpoint junction in the t(11;18) (q21;q21) translocation of a gastric MALT lymphoma. *Biochem. Biophys. Res. Commun.* **2001**, *280*: 301-306.